APPENDICES

Page

A. Independence, Randomization, and Outliers 566

1. Statistical Independence 566
2. Randomization 566
3 Outliers 572

B. Validating Normality and Homogeneity of Variance Assumptions 575

1. Introduction 575
2. Tests for Normal Distribution of Data 575
3. Test for Homogeneity of Variance 582
4. Transformations of the Data 584

C. Dunnett's Procedure 587

1. Manual Calculations 587
2. Computer Calculations 595

D. *t* test with the Bonferroni Adjustment 602

E. Steel's Many-one Rank Test 609

F. Wilcoxon Rank Sum Test with the Bonferroni Adjustment 615

G. Single Concentration Toxicity Test - Comparison of Control with 100% Effluent or Receiving Water or Comparison of Dilution and Controls 622

H. Probit Analysis 627

I. Spearman-Karber Method 631

J. Trimmed Spearman-Karber Method 638

K. Graphical Method 643

L. Linear Interpolation Method 648

1. General Procedure 648
2. Data Summary and Plots 648
3. Monotonicity 648
4. Linear Interpolation Method 649
5. Confidence Intervals 650
6. Manual Calculations 651
7. Computer Calculations 655

Cited References 659

APPENDIX A

INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

1. STATISTICAL INDEPENDENCE

1.1 Dunnett's Procedure and the *t* test with Bonferroni's adjustment are parametric procedures based on the assumptions that (1) the observations within treatments are independent and normally distributed, and (2) that the variance of the observations is homogeneous across all toxicant concentrations and the control. Of the three possible departures from the assumptions, non-normality, heterogeneity of variance, and lack of independence, those caused by lack of independence are the most difficult to resolve (see Scheffe, 1959). For toxicity data, statistical independence means that given knowledge of the true mean for a given concentration or control, knowledge of the error in any one actual observation would provide no information about the error in any other observation. Lack of independence is difficult to assess and difficult to test for statistically. It may also have serious effects on the true alpha or beta level. Therefore, it is of utmost importance to be aware of the need for statistical independence between observations and to be constantly vigilant in avoiding any patterned experimental procedure that might compromise independence. One of the best ways to help insure independence is to follow proper randomization procedures throughout the test.

2. RANDOMIZATION

2.1 Randomization of the distribution of test organisms among test chambers, and the arrangement of treatments and replicate chambers is an important part of conducting a valid test. The purpose of randomization is to avoid situations where test organisms are placed serially into test chambers, or where all replicates for a test concentration are located adjacent to one another, which could introduce bias into the test results.

2.2 An example of randomization of the distribution of test organisms among test chambers, and an example of randomization of arrangement of treatments and replicate chambers are described using the topsmelt, *Atherinops affinis*, Survival and Growth test. For the purpose of the example, the test design is as follows:

Five effluent concentrations are tested in addition to the control. The effluent concentrations are as follows: 6.25%, 12.5%, 25.0%, 50.0%, and 100.0%. There are five replicate chambers per treatment. Each replicate chamber contains five larvae.

2.3 RANDOMIZATION OF FISH TO REPLICATE CHAMBERS EXAMPLE

2.3.1 Consider first the random assignment of the fish to the replicate chambers. The first step is to label each of the replicate chambers with the control or effluent concentration and the replicate number. The next step is to assign each replicate chamber three double-digit numbers. An example of this assignment is provided in Table A.1. Note that the double digits 00 and 91 through 99 were not used.

2.3.2 The random numbers used to carry out the random assignment of fish to replicate chambers are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double digit number. The first number read identifies the replicate chamber for the first fish taken from the tank. For the example, the first entry in row 2 was chosen as the starting position. The first number in this row is 37. According to Table A.1, this number corresponds to replicate chamber 2 of the 6.25% effluent concentration. Thus, the first fish taken from the tank is to be placed in replicate chamber 2 of the 6.25% effluent concentration.

2.3.3 The next step is to read the double digit number to the right of the first one. The second number identifies the replicate chamber for the second fish taken from the tank. Continuing the example, the second number read in row 2 of Table A.2 is 54. According to Table A.1, this number corresponds to replicate chamber 4 of the 50.0% effluent concentration. Thus, the second fish taken from the tank is to be placed in replicate chamber 4 of the 50.0% effluent concentration.

2.3.4 Continue in this fashion until all the fish have been randomly assigned to a replicate chamber. In order to fill each replicate chamber with ten fish, the assigned numbers will be used more than once. If a number is read from the table that was not assigned to a replicate chamber, then ignore it and continue to the next number. If a replicate chamber becomes filled and a

number is read from the table that corresponds to it, then ignore that value and continue to the next number. The first ten random summarized in Table A.3.2.3.5 Three double-digit numbers were assigned to each replicate chamber (instead of one or two double-digit numbers) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each replicate chamber: the first column of assigned numbers in Table A.1. Whenever the numbers 00 and 31 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

TABLE A.1. RANDOM ASSIGNMENT OF FISH TO REPLICATE CHAMBERS EXAMPLE
ASSIGNED NUMBERS FOR EACH REPLICATE CHAMBER

Assigned Numbers	Replicate Chamber	
01, 31, 61	Control,	replicate chamber 1
02, 32, 62	Control,	replicate chamber 2
03, 33, 63	Control,	replicate chamber 3
04, 34, 64	Control,	replicate chamber 4
05, 35, 65	Control,	replicate chamber 5
06, 36, 66	6.25% effluent,	replicate chamber 1
07, 37, 67	6.25% effluent,	replicate chamber 2
08, 38, 68	6.25% effluent,	replicate chamber 3
09, 39, 69	6.25% effluent,	replicate chamber 4
10, 40, 70	6.25% effluent,	replicate chamber 5
11, 41, 71	12.5% effluent,	replicate chamber 1
12, 42, 72	12.5% effluent,	replicate chamber 2
13, 43, 73	12.5% effluent,	replicate chamber 3
14, 44, 74	12.5% effluent,	replicate chamber 4
15, 45, 75	12.5% effluent,	replicate chamber 5
16, 46, 76	25.0% effluent,	replicate chamber 1
17, 47, 77	25.0% effluent,	replicate chamber 2
18, 48, 78	25.0% effluent,	replicate chamber 3
19, 49, 79	25.0% effluent,	replicate chamber 4
20, 50, 80	25.0% effluent,	replicate chamber 5
21, 51, 81	50.0% effluent,	replicate chamber 1
22, 52, 82	50.0% effluent,	replicate chamber 2
23, 53, 83	50.0% effluent,	replicate chamber 3
24, 54, 84	50.0% effluent,	replicate chamber 4
25, 55, 85	50.0% effluent,	replicate chamber 5
26, 56, 86	100.0% effluent,	replicate chamber 1
27, 57, 87	100.0% effluent,	replicate chamber 2
28, 58, 88	100.0% effluent,	replicate chamber 3
29, 59, 89	100.0% effluent,	replicate chamber 4
30, 60, 90	100.0% effluent,	replicate chamber 5

TABLE A.2. TABLE OF RANDOM NUMBERS (Dixon and Massey, 1983)

10 09 73 25 33	76 52 01 35 86	34 67 35 43 76	80 95 90 91 17	39 29 27 49 45
37 54 20 48 05	64 89 47 42 96	24 80 52 40 37	20 63 61 04 02	00 82 29 16 65
08 42 26 89 53	19 64 50 93 03	23 20 90 25 60	15 95 33 47 64	35 08 03 36 06
99 01 90 25 29	09 37 67 07 15	38 31 13 11 65	88 67 67 43 97	04 43 62 76 59
12 80 79 99 70	80 15 73 61 47	64 03 23 66 53	98 95 11 68 77	12 27 17 68 33
66 06 57 47 17	34 07 27 68 50	36 69 73 61 70	65 81 33 98 85	11 19 92 91 70
31 06 01 08 05	45 57 18 24 06	35 30 34 26 14	86 79 90 74 39	23 40 30 97 32
85 26 97 76 02	02 05 16 56 92	68 66 57 48 18	73 05 38 52 47	18 62 38 85 79
63 57 33 21 35	05 32 54 70 48	90 55 35 75 48	28 46 82 87 09	83 49 12 56 24
73 79 64 57 53	03 52 96 47 78	35 80 83 42 82	60 93 52 03 44	35 27 38 84 35
98 52 01 77 67	14 90 56 86 07	22 10 94 05 58	60 97 09 34 33	50 50 07 39 98
11 80 50 54 31	39 80 82 77 32	50 72 56 82 48	29 40 52 42 01	52 77 56 78 51
83 45 29 96 34	06 28 89 80 83	13 74 67 00 78	18 47 54 06 10	68 71 17 78 17
88 68 54 02 00	86 50 75 84 01	36 76 66 79 51	90 36 47 64 93	29 60 91 10 62
99 59 46 73 48	87 51 76 49 69	91 82 60 89 28	93 78 56 13 68	23 47 83 41 13
65 48 11 76 74	17 46 85 09 50	58 04 77 69 74	73 03 95 71 86	40 21 81 65 44
80 12 43 56 35	17 72 70 80 15	45 31 82 23 74	21 11 57 82 53	14 38 55 37 63
74 35 09 98 17	77 40 27 72 14	43 23 60 02 10	45 52 16 42 37	96 28 60 26 55
69 91 62 68 03	66 25 22 91 48	36 93 68 72 03	76 62 11 39 90	94 40 05 64 18
09 89 32 05 05	14 22 56 85 14	46 42 75 67 88	96 29 77 88 22	54 38 21 45 98
91 49 91 45 23	68 47 92 76 86	46 16 28 35 54	94 75 08 99 23	37 08 92 00 48
80 33 69 45 98	26 94 03 68 58	70 29 73 41 35	53 14 03 33 40	42 05 08 23 41
44 10 48 19 49	85 15 74 79 54	32 97 92 65 75	57 60 04 08 81	22 22 20 64 13
12 55 07 37 42	11 10 00 20 40	12 86 07 46 97	96 64 48 94 39	28 70 72 58 15
63 60 64 93 29	16 50 53 44 84	40 21 95 25 63	43 65 17 70 82	07 20 73 17 90
61 19 69 04 46	26 45 74 77 74	51 92 43 37 29	65 39 45 95 93	42 58 26 05 27
15 47 44 52 66	95 27 07 99 53	59 36 78 38 48	82 39 61 01 18	33 21 15 94 66
94 55 72 85 73	67 89 75 43 87	54 62 24 44 31	91 19 04 25 92	92 92 74 59 73
42 48 11 62 13	97 34 40 87 21	16 86 84 87 67	03 07 11 20 59	25 70 14 66 70
23 52 37 83 17	73 20 88 98 37	68 93 59 14 16	26 25 22 96 63	05 52 28 25 62
04 49 35 24 94	75 24 63 38 24	45 86 25 10 25	61 96 27 93 35	65 33 71 24 72
00 54 99 76 54	64 05 18 81 59	96 11 96 38 96	54 69 28 23 91	23 28 72 95 29
35 96 31 53 07	26 89 80 93 45	33 35 13 54 62	77 97 45 00 24	90 10 33 93 33
59 80 80 83 91	45 42 72 68 42	83 60 94 97 00	13 02 12 48 92	78 56 52 01 06
46 05 88 52 36	01 39 09 22 86	77 28 14 40 77	93 91 08 36 47	70 61 74 29 41
32 17 90 05 97	87 37 92 52 41	05 56 70 70 07	86 74 31 71 57	85 39 41 18 38
69 23 46 14 06	20 11 74 52 04	15 95 66 00 00	18 74 39 24 23	97 11 89 63 38
19 56 54 14 30	01 75 87 53 79	40 41 92 15 85	66 67 43 68 06	84 96 28 52 07
45 15 51 49 38	19 47 60 72 46	43 66 79 45 43	59 04 79 00 33	20 82 66 95 41
94 86 43 19 94	36 16 81 08 51	34 88 88 15 53	01 54 03 54 56	05 01 45 11 76
98 08 62 48 26	45 24 02 84 04	44 99 90 88 96	39 09 47 34 07	35 44 13 18 80
33 18 51 62 32	41 94 15 09 49	89 43 54 85 81	88 69 54 19 94	37 54 87 30 43
80 95 10 04 06	96 38 27 07 74	20 15 12 33 87	25 01 62 52 98	94 62 46 11 71
79 75 24 91 40	71 96 12 82 96	69 86 10 25 91	74 85 22 05 39	00 38 75 95 79
18 63 33 25 37	98 14 50 65 71	31 01 02 46 74	05 45 56 14 27	77 93 89 19 36
74 02 94 39 02	77 55 73 22 70	97 79 01 71 19	52 52 75 80 21	80 81 45 17 48
54 17 84 56 11	80 99 33 71 43	05 33 51 29 69	56 12 71 92 55	36 04 09 03 24
11 66 44 98 83	52 07 98 48 27	59 38 17 15 39	09 97 33 34 40	88 46 12 33 56
48 32 47 79 28	31 24 96 47 10	02 29 53 68 70	32 30 75 75 46	15 02 00 99 94
69 07 49 41 38	87 63 79 19 76	35 58 40 44 01	10 51 82 16 15	01 84 87 69 38

2.4 RANDOMIZATION OF REPLICATE CHAMBERS TO POSITIONS EXAMPLE

2.4.1 Next consider the random assignment of the 30 replicate chambers to positions within the water bath (or equivalent). Assume that the replicate chambers are to be positioned in a five row by six column rectangular array. The first step is to label the positions in the water bath. Table A.4 provides an example layout. assignments of fish to replicate chambers for the example are

TABLE A.3. EXAMPLE OF RANDOM ASSIGNMENT OF FIRST TEN FISH TO REPLICATE CHAMBERS

Fish	Assignment
First fish taken from tank	6.25% effluent, replicate chamber 2
Second fish taken from tank	50.0% effluent, replicate chamber 4
Third fish taken from tank	25.0% effluent, replicate chamber 5
Fourth fish taken from tank	25.0% effluent, replicate chamber 3
Fifth fish taken from tank	Control, replicate chamber 5
Sixth fish taken from tank	Control, replicate chamber 4
Seventh fish taken from tank	100.0% effluent, replicate chamber 4
Eighth fish taken from tank	25.0% effluent, replicate chamber 2
Ninth fish taken from tank	12.5% effluent, replicate chamber 2
Tenth fish taken from tank	50.0% effluent, replicate chamber 4

TABLE A.4. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE LABELLING THE POSITIONS WITHIN THE WATER BATH

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24
25	26	27	28	29	30

2.4.2 The second step is to assign each of the 30 positions three double-digit numbers. An example of this assignment is provided in Table A.5. Note that the double digits 00 and 91 through 99 were not used.

2.4.3 The random numbers used to carry out the random assignment of replicate chambers to positions are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double-digit number. The first number read identifies the position for the first replicate chamber of the control. For the example, the first entry in row 10 of Table A.2 was chosen as the starting position. The first number in this row was 73. According to Table A.5, this number corresponds to position 13. Thus, the first replicate chamber for the control will be placed in position 13.

2.4.4 The next step is to read the double-digit number to the right of the first one. The second number identifies the position for the second replicate chamber of the control. Continuing the example, the second number read in row 10 of Table A.2 is 79. According to Table A.5, this number corresponds to position 19. Thus, the second replicate chamber for the control will be placed in position 19.

2.4.5 Continue in this fashion until all the replicate chambers have been assigned to a position. The first five numbers read will identify the positions for the control replicate chambers, the second five numbers read will identify the positions for the lowest effluent concentration replicate chambers, and so on. If a number is read from the table that was not assigned to a position, then ignore that value and continue to the next number. If a number is repeated in Table A.2, then ignore the repeats and continue to the next number. The complete randomization of replicate chambers to positions for the example is displayed in Table A.6.

2.4.6 Three double-digit numbers were assigned to each position (instead of one or two) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each position: the first column of assigned numbers in Table A.5. Whenever the numbers 00 and 31 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

3. **OUTLIERS**

3.1 An outlier is an inconsistent or questionable data point that appears unrepresentative of the general trend exhibited by the majority of the data. Outliers may be detected by tabulation of the data, plotting, and by an analysis of the residuals. An explanation should be sought for any questionable data points. Without an explanation, data points should be discarded only with extreme caution. If there is no explanation, the analysis should be performed both with and without the outlier, and the results of both analyses should be reported.

3.2 Gentleman-Wilk's A statistic gives a test for the condition that the extreme observation may be considered an outlier. For a discussion of this, and other techniques for evaluating outliers, see Draper and John (1981).

TABLE *A.5.* *RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE ASSIGNED NUMBERS FOR EACH POSITION*

Assigned Numbers	*Position*
01, 31, 61	*1*
02, 32, 62	*2*
03, 33, 63	*3*
04, 34, 64	*4*
05, 35, 65	*5*
06, 36, 66	*6*
07, 37, 67	*7*
08, 38, 68	*8*
09, 39, 69	*9*
10, 40, 70	*10*
11, 41, 71	*11*
12, 42, 72	*12*
13, 43, 73	*13*
14, 44, 74	*14*
15, 45, 75	*15*
16, 46, 76	*16*
17, 47, 77	*17*
18, 48, 78	*18*
19, 49, 79	*19*
20, 50, 80	*20*
21, 51, 81	*21*
22, 52, 82	*22*
23, 53, 83	*23*
24, 54, 84	*24*
25, 55, 85	*25*
26, 56, 86	*26*
27, 57, 87	*27*
28, 58, 88	*28*
29, 59, 89	*29*
30, 60, 90	*30*

TABLE A.6. EXAMPLE OF RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS:

ASSIGNMENT OF ALL 30 POSITIONS

25.0%	50.0%	6.25%	Control	6.25%	100.0%
25.0%	12.5%	50.0%	25.0%	50.0%	12.5%
Control	12.5%	100.0%	100.0%	6.25%	6.25%
Control	12.5%	100.0%	6.25%	Control	25.0%
100.0%	25.0%	Control	50.0%	50.0%	12.5%

APPENDIX B

VALIDATING NORMALITY AND HOMOGENEITY OF VARIANCE ASSUMPTIONS

1. INTRODUCTION

1.1 Dunnett's Procedure and the *t* test with Bonferroni's adjustment are parametric procedures based on the assumptions that the observations within treatments are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using these tests, to determine if they have been met. Tests for validating the assumptions are provided in the following discussion. If the tests fail (if the data do not meet the assumptions), a nonparametric procedure such as Steel's Many-one Rank Test may be more appropriate. However, the decision on whether to use parametric or nonparametric tests may be a judgement call, and a statistician should be consulted in selecting the analysis.

2. TEST FOR NORMAL DISTRIBUTION OF DATA

2.1 SHAPIRO-WILK'S TEST

2.1.1 One formal test for normality is the Shapiro-Wilk's Test (Conover, 1980). The test statistic is obtained by dividing the square of an appropriate linear combination of the sample order statistics by the usual symmetric estimate of variance. The calculated W must be greater than zero and less than or equal to one. This test is recommended for a sample size of 50 or less. If the sample size is greater than 50, the Kolmogorov "D" statistic (Stephens, 1974) is recommended. An example of the Shapiro-Wilk's test is provided below.

2.2 The example uses growth data from the Mysid Larval Survival and Growth Test. The same data are used later in the discussions of the homogeneity of variance determination in Section 3 of this appendix and Dunnett's Procedure in Appendix C. The data, the mean and variance of the observations at each concentration, including the control, are listed in Table B.1.

TABLE B.1. MYSID, *HOLMESIMYSIS COSTATA*, GROWTH DATA

Replicate	Control	Concentration (%) 1.80	3.20	5.60
1	0.048	0.055	0.057	0.041
2	0.058	0.048	0.050	0.040
3	0.047	0.042	0.046	0.041
4	0.058	0.041	0.043	0.043
5	0.051	0.052	0.045	0.040
Mean($\bar{X}_i$)	0.052	0.048	0.048	0.041
S_i^2	0.0000283	0.0000373	0.0000307	0.0000015
i	1	2	3	4

2.3 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table B.2.

TABLE B.2. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	Concentration (%) 1.80	3.20	5.60
1	-0.004	0.007	0.009	0.000
2	0.006	0.000	0.002	-0.001
3	-0.005	-0.006	-0.002	0.000
4	0.006	-0.007	-0.005	0.002
5	-0.001	0.004	-0.003	-0.001

2.4 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

& = the overall mean of the centered observations

n = the total number of centered observations

2.4.1 For this set of data, n = 20

$$\& = \frac{1}{20}(0.001) = 0.000$$

$$D = 0.000393$$

2.5 Order the centered observations from smallest to largest

$$X^{(1)} \# X^{(2)} \# \ldots \# X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table B.3.

TABLE B.3. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.007	11	0.000
2	-0.006	12	0.000
3	-0.005	13	0.000
4	-0.005	14	0.002
5	-0.004	15	0.002
6	-0.003	16	0.004
7	-0.002	17	0.006
8	-0.001	18	0.006
9	-0.001	19	0.007
10	-0.001	20	0.009

2.6 From Table B.4, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table B.5.

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST (Conover, 1980)

	Number of Observations								
$i \pm^n$	2	3	4	5	6	7	8	9	10
1	0.7071	0.7071	0.6872	0.6646	0.6431	0.6233	0.6052	0.5888	0.5739
2	-	0.0000	0.1667	0.2413	0.2806	0.3031	0.3164	0.3244	0.3291
3	-	-	-	0.0000	0.0875	0.1401	0.1743	0.1976	0.2141
4	-	-	-	-	-	0.0000	0.0561	0.0947	0.1224
5	-	-	-	-	-	-	-	0.0000	0.0399

	Number of Observations									
$i \pm^n$	11	12	13	14	15	16	17	18	19	20
1	0.5601	0.5475	0.5359	0.5251	0.5150	0.5056	0.4968	0.4886	0.4808	0.4734
2	0.3315	0.3325	0.3325	0.3318	0.3306	0.3290	0.3273	0.3253	0.3232	0.3211
3	0.2260	0.2347	0.2412	0.2460	0.2495	0.2521	0.2540	0.2553	0.2561	0.2565
4	0.1429	0.1586	0.1707	0.1802	0.1878	0.1939	0.1988	0.2027	0.2059	0.2085
5	0.0695	0.0922	0.1099	0.1240	0.1353	0.1447	0.1524	0.1587	0.1641	0.1686
6	0.0000	0.0303	0.0539	0.0727	0.0880	0.1005	0.1109	0.1197	0.1271	0.1334
7	-	-	0.0000	0.0240	0.0433	0.0593	0.0725	0.0837	0.0932	0.1013
8	-	-	-	-	0.0000	0.0196	0.0359	0.0496	0.0612	0.0711
9	-	-	-	-	-	-	0.0000	0.0163	0.0303	0.0422
10	-	-	-	-	-	-	-	-	0.0000	0.0140

	Number of Observations									
$i \pm^n$	21	22	23	24	25	26	27	28	29	30
1	0.4643	0.4590	0.4542	0.4493	0.4450	0.4407	0.4366	0.4328	0.4291	0.4254
2	0.3185	0.3156	0.3126	0.3098	0.3069	0.3043	0.3018	0.2992	0.2968	0.2944
3	0.2578	0.2571	0.2563	0.2554	0.2543	0.2533	0.2522	0.2510	0.2499	0.2487
4	0.2119	0.2131	0.2139	0.2145	0.2148	0.2151	0.2152	0.2151	0.2150	0.2148
5	0.1736	0.1764	0.1787	0.1807	0.1822	0.1836	0.1848	0.1857	0.1864	0.1870
6	0.1399	0.1443	0.1480	0.1512	0.1539	0.1563	0.1584	0.1601	0.1616	0.1630
7	0.1092	0.1150	0.1201	0.1245	0.1283	0.1316	0.1346	0.1372	0.1395	0.1415
8	0.0804	0.0878	0.0941	0.0997	0.1046	0.1089	0.1128	0.1162	0.1192	0.1219
9	0.0530	0.0618	0.0696	0.0764	0.0823	0.0876	0.0923	0.0965	0.1002	0.1036
10	0.0263	0.0368	0.0459	0.0539	0.0610	0.0672	0.0728	0.0778	0.0822	0.0862
11	0.0000	0.0122	0.0228	0.0321	0.0403	0.0476	0.0540	0.0598	0.0650	0.0697
12	-	-	0.0000	0.0107	0.0200	0.0284	0.0358	0.0424	0.0483	0.0537
13	-	-	-	-	0.0000	0.0094	0.0178	0.0253	0.0320	0.0381
14	-	-	-	-	-	-	0.0000	0.0084	0.0159	0.0227
15	-	-	-	-	-	-	-	-	0.0000	0.0076

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST (CONTINUED)

	Number of Observations									
$i \pm^n$	31	32	33	34	35	36	37	38	39	40
1	0.4220	0.4188	0.4156	0.4127	0.4096	0.4068	0.4040	0.4015	0.3989	0.3964
2	0.2921	0.2898	0.2876	0.2854	0.2834	0.2813	0.2794	0.2774	0.2755	0.2737
3	0.2475	0.2462	0.2451	0.2439	0.2427	0.2415	0.2403	0.2391	0.2380	0.2368
4	0.2145	0.2141	0.2137	0.2132	0.2127	0.2121	0.2116	0.2110	0.2104	0.2098
5	0.1874	0.1878	0.1880	0.1882	0.1883	0.1883	0.1883	0.1881	0.1880	0.1878
6	0.1641	0.1651	0.1660	0.1667	0.1673	0.1678	0.1683	0.1686	0.1689	0.1691
7	0.1433	0.1449	0.1463	0.1475	0.1487	0.1496	0.1505	0.1513	0.1520	0.1526
8	0.1243	0.1265	0.1284	0.1301	0.1317	0.1331	0.1344	0.1356	0.1366	0.1376
9	0.1066	0.1093	0.1118	0.1140	0.1160	0.1179	0.1196	0.1211	0.1225	0.1237
10	0.0899	0.0931	0.0961	0.0988	0.1013	0.1036	0.1056	0.1075	0.1092	0.1108
11	0.0739	0.0777	0.0812	0.0844	0.0873	0.0900	0.0924	0.0947	0.0967	0.0986
12	0.0585	0.0629	0.0669	0.0706	0.0739	0.0770	0.0798	0.0824	0.0848	0.0870
13	0.0435	0.0485	0.0530	0.0572	0.0610	0.0645	0.0677	0.0706	0.0733	0.0759
14	0.0289	0.0344	0.0395	0.0441	0.0484	0.0523	0.0559	0.0592	0.0622	0.0651
15	0.0144	0.0206	0.0262	0.0314	0.0361	0.0404	0.0444	0.0481	0.0515	0.0546
16	0.0000	0.0068	0.0131	0.0187	0.0239	0.0287	0.0331	0.0372	0.0409	0.0444
17	-	-	0.0000	0.0062	0.0119	0.0172	0.0220	0.0264	0.0305	0.0343
18	-	-	-	-	0.0000	0.0057	0.0110	0.0158	0.0203	0.0244
19	-	-	-	-	-	-	0.0000	0.0053	0.0101	0.0146
20	-	-	-	-	-	-	-	-	0.0000	0.0049

	Number of Observations									
$i \pm^n$	41	42	43	44	45	46	47	48	49	50
1	0.3940	0.3917	0.3894	0.3872	0.3850	0.3830	0.3808	0.3789	0.3770	0.3751
2	0.2719	0.2701	0.2684	0.2667	0.2651	0.2635	0.2620	0.2604	0.2589	0.2574
3	0.2357	0.2345	0.2334	0.2323	0.2313	0.2302	0.2291	0.2281	0.2271	0.2260
4	0.2091	0.2085	0.2078	0.2072	0.2065	0.2058	0.2052	0.2045	0.2038	0.2032
5	0.1876	0.1874	0.1871	0.1868	0.1865	0.1862	0.1859	0.1855	0.1851	0.1847
6	0.1693	0.1694	0.1695	0.1695	0.1695	0.1695	0.1695	0.1693	0.1692	0.1691
7	0.1531	0.1535	0.1539	0.1542	0.1545	0.1548	0.1550	0.1551	0.1553	0.1554
8	0.1384	0.1392	0.1398	0.1405	0.1410	0.1415	0.1420	0.1423	0.1427	0.1430
9	0.1249	0.1259	0.1269	0.1278	0.1286	0.1293	0.1300	0.1306	0.1312	0.1317
10	0.1123	0.1136	0.1149	0.1160	0.1170	0.1180	0.1189	0.1197	0.1205	0.1212
11	0.1004	0.1020	0.1035	0.1049	0.1062	0.1073	0.1085	0.1095	0.1105	0.1113
12	0.0891	0.0909	0.0927	0.0943	0.0959	0.0972	0.0986	0.0998	0.1010	0.1020
13	0.0782	0.0804	0.0824	0.0842	0.0860	0.0876	0.0892	0.0906	0.0919	0.0932
14	0.0677	0.0701	0.0724	0.0745	0.0765	0.0783	0.0801	0.0817	0.0832	0.0846
15	0.0575	0.0602	0.0628	0.0651	0.0673	0.0694	0.0713	0.0731	0.0748	0.0764
16	0.0476	0.0506	0.0534	0.0560	0.0584	0.0607	0.0628	0.0648	0.0667	0.0685
17	0.0379	0.0411	0.0442	0.0471	0.0497	0.0522	0.0546	0.0568	0.0588	0.0608
18	0.0283	0.0318	0.0352	0.0383	0.0412	0.0439	0.0465	0.0489	0.0511	0.0532
19	0.0188	0.0227	0.0263	0.0296	0.0328	0.0357	0.0385	0.0411	0.0436	0.0459
20	0.0094	0.0136	0.0175	0.0211	0.0245	0.0277	0.0307	0.0335	0.0361	0.0386
21	0.0000	0.0045	0.0087	0.0126	0.0163	0.0197	0.0229	0.0259	0.0288	0.0314
22	-	-	0.0000	0.0042	0.0081	0.0118	0.0153	0.0185	0.0215	0.0244
23	-	-	-	-	0.0000	0.0039	0.0076	0.0111	0.0143	0.0174
24	-	-	-	-	-	-	0.0000	0.0037	0.0071	0.0104
25	-	-	-	-	-	-	-	-	0.0000	0.0035

2.7 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table B.5. For this set of data:

$$W = \frac{1}{0.000393}(0.0194)^2 = 0.958$$

TABLE B.5. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.016	$X^{(20)} - X^{(1)}$
2	0.3211	0.013	$X^{(19)} - X^{(2)}$
3	0.2565	0.011	$X^{(18)} - X^{(3)}$
4	0.2085	0.011	$X^{(17)} - X^{(4)}$
5	0.1686	0.008	$X^{(16)} - X^{(5)}$
6	0.1334	0.005	$X^{(15)} - X^{(6)}$
7	0.1013	0.004	$X^{(14)} - X^{(7)}$
8	0.0711	0.001	$X^{(13)} - X^{(8)}$
9	0.0422	0.001	$X^{(12)} - X^{(9)}$
10	0.0140	0.001	$X^{(11)} - X^{(10)}$

2.8 The decision rule for this test is to compare the computed W to the critical value found in Table B.6. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 20 observations is 0.868. Since W = 0.958 is greater than the critical value, conclude that the data are normally distributed.

2.9 In general, if the data fail the test for normality, a transformation such as to log values may normalize the data. After transforming the data, repeat the Shapiro Wilk's Test for normality.

TABLE B.6. QUANTILES OF THE SHAPIRO WILK'S TEST STATISTIC (Conover, 1980)

n	0.01	0.02	0.05	0.10	0.50	0.90	0.95	0.98	0.99
3	0.753	0.756	0.767	0.789	0.959	0.998	0.999	1.000	1.000
4	0.687	0.707	0.748	0.792	0.935	0.987	0.992	0.996	0.997
5	0.686	0.715	0.762	0.806	0.927	0.979	0.986	0.991	0.993
6	0.713	0.743	0.788	0.826	0.927	0.974	0.981	0.986	0.989
7	0.730	0.760	0.803	0.838	0.928	0.972	0.979	0.985	0.988
8	0.749	0.778	0.818	0.851	0.932	0.972	0.978	0.984	0.987
9	0.764	0.791	0.829	0.859	0.935	0.972	0.978	0.984	0.986
10	0.781	0.806	0.842	0.869	0.938	0.972	0.978	0.983	0.986
11	0.792	0.817	0.850	0.876	0.940	0.973	0.979	0.984	0.986
12	0.805	0.828	0.859	0.883	0.943	0.973	0.979	0.984	0.986
13	0.814	0.837	0.866	0.889	0.945	0.974	0.979	0.984	0.986
14	0.825	0.846	0.874	0.895	0.947	0.975	0.980	0.984	0.986
15	0.835	0.855	0.881	0.901	0.950	0.975	0.980	0.984	0.987
16	0.844	0.863	0.887	0.906	0.952	0.976	0.981	0.985	0.987
17	0.851	0.869	0.892	0.910	0.954	0.977	0.981	0.985	0.987
18	0.858	0.874	0.897	0.914	0.956	0.978	0.982	0.986	0.988
19	0.863	0.879	0.901	0.917	0.957	0.978	0.982	0.986	0.988
20	0.868	0.884	0.905	0.920	0.959	0.979	0.983	0.986	0.988
21	0.873	0.888	0.908	0.923	0.960	0.980	0.983	0.987	0.989
22	0.878	0.892	0.911	0.926	0.961	0.980	0.984	0.987	0.989
23	0.881	0.895	0.914	0.928	0.962	0.981	0.984	0.987	0.989
24	0.884	0.898	0.916	0.930	0.963	0.981	0.984	0.987	0.989
25	0.888	0.901	0.918	0.931	0.964	0.981	0.985	0.988	0.989
26	0.891	0.904	0.920	0.933	0.965	0.982	0.985	0.988	0.989
27	0.894	0.906	0.923	0.935	0.965	0.982	0.985	0.988	0.990
28	0.896	0.908	0.924	0.936	0.966	0.982	0.985	0.988	0.990
29	0.898	0.910	0.926	0.937	0.966	0.982	0.985	0.988	0.990
30	0.900	0.912	0.927	0.939	0.967	0.983	0.985	0.988	0.990
31	0.902	0.914	0.929	0.940	0.967	0.983	0.986	0.988	0.990
32	0.904	0.915	0.930	0.941	0.968	0.983	0.986	0.988	0.990
33	0.906	0.917	0.931	0.942	0.968	0.983	0.986	0.989	0.990
34	0.908	0.919	0.933	0.943	0.969	0.983	0.986	0.989	0.990
35	0.910	0.920	0.934	0.944	0.969	0.984	0.986	0.989	0.990
36	0.912	0.922	0.935	0.945	0.970	0.984	0.986	0.989	0.990
37	0.914	0.924	0.936	0.946	0.970	0.984	0.987	0.989	0.990
38	0.916	0.925	0.938	0.947	0.971	0.984	0.987	0.989	0.990
39	0.917	0.927	0.939	0.948	0.971	0.984	0.987	0.989	0.991
40	0.919	0.928	0.940	0.949	0.972	0.985	0.987	0.989	0.991
41	0.920	0.929	0.941	0.950	0.972	0.985	0.987	0.989	0.991
42	0.922	0.930	0.942	0.951	0.972	0.985	0.987	0.989	0.991
43	0.923	0.932	0.943	0.951	0.973	0.985	0.987	0.990	0.991
44	0.924	0.933	0.944	0.952	0.973	0.985	0.987	0.990	0.991
45	0.926	0.934	0.945	0.953	0.973	0.985	0.988	0.990	0.991
46	0.927	0.935	0.945	0.953	0.974	0.985	0.988	0.990	0.991
47	0.928	0.936	0.946	0.954	0.974	0.985	0.988	0.990	0.991
48	0.929	0.937	0.947	0.954	0.974	0.985	0.988	0.990	0.991
49	0.929	0.937	0.947	0.955	0.974	0.985	0.988	0.990	0.991
50	0.930	0.938	0.947	0.955	0.974	0.985	0.988	0.990	0.991

3. **TEST FOR HOMOGENEITY OF VARIANCE**

3.1 For Dunnett's Procedure and the t test with Bonferroni's adjustment, the variances of the data obtained from each toxicant concentration and the control are assumed to be equal. Bartlett's Test is a formal test of this assumption. In using this test, it is assumed that the data are normally distributed.

3.2 The data used in this example are growth data from a Mysid Survival and Growth Test, and are the same data used in Appendix C. These data are listed in Table B.7, together with the calculated variance for the control and each toxicant concentration.

TABLE B.7. MYSID, *HOLMESIMYSIS COSTATA*, GROWTH DATA

		Concentration (%)		
Replicate	Control	1.80	3.20	5.60
1	0.048	0.055	0.057	0.041
2	0.058	0.048	0.050	0.040
3	0.047	0.042	0.046	0.041
4	0.058	0.041	0.043	0.043
5	0.051	0.052	0.045	0.040
Mean($\bar{X}_i$)	0.052	0.048	0.048	0.041
S_i^2	0.0000283	0.0000373	0.0000307	0.0000015
i	1	2	3	4

3.3 The test statistic for Bartlett's Test (Snedecor and Cochran, 1980) is as follows:

$$B = \frac{\left[\left(\sum_{i=1}^{p} V_i\right) \ln \bar{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2\right]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, ($V_i = n_i - 1$)

p = number of levels of toxicant concentration including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

n_i =the number of replicates for concentration i.

$$\bar{S}^2 = \frac{\left(\sum_{i=1}^{p} V_i S_i^2\right)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + [3(p-1)]^{-1}\left[\sum_{i=1}^{p} 1/V_i - \left(\sum_{i=1}^{p} V_i\right)^{-1}\right]$$

3.4 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value is obtained from a table of the chi-square distribution for p - 1 degrees of freedom and a significance level of 0.01. If B is less than the critical value then the variances are assumed to be equal.

3.5 For the data in this example, all concentrations including the control have the same number of replicates (n_i - 5 for all i). Thus, V_i = 4 for all i. For this data, p = 4, $\bar{S}^2$ = 0.0000245, and C = 1.104. Bartlett's statistic is therefore:

$$B = [(16)\ln(0.0000245) - 4\sum_{i=1}^{p} \ln(S_i^2)]/1.104$$

$$= [16(-10.617) - 4(-44.470)]/1.104$$

$$= [-169.872 - (-177.880)]/1.104$$

$$= 7.254$$

3.6 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value for the test is 9.21 for a significance level of 0.01. Since B = 7.254 is less than 9.21, conclude that the variances are not different.

4. **TRANSFORMATIONS OF THE DATA**

4.1 When the assumptions of normality and/or homogeneity of variance are not met, transformations of the data may remedy the problem, so that the data can be analyzed by parametric procedures, rather than nonparametric technique such as Steel's Many-one Rank Test or Wilcoxon's Rank Sum Test. Examples of transformations include log, square root, arc sine square root, and reciprocals. After the data have been transformed, the Shapiro-Wilk's and Bartlett's tests should be performed on the transformed observations to determine whether the assumptions of normality and/or homogeneity of variance are met.

4.2 ARC SINE SQUARE ROOT TRANSFORMATION (USEPA, 1993).

4.2.1 For data consisting of proportions from a binomial (response/no response; live/dead) response variable, the variance within the ith treatment is proportional to P_i (1 - P_i), where P_i is the expected proportion for the treatment. This clearly violates the homogeneity of variance assumption required by parametric procedures such as Dunnett's Procedure or the *t* test with Bonferroni's adjustment, since the existence of a treatment effect implies different values of P_i for different treatments, i. Also, when the observed proportions are based on small samples, or when P_i is close to zero or one, the normality assumption may be invalid. The arc sine square root (arc sine **%&&&**) transformation is commonly used for such data to stabilize the variance and satisfy the normality requirement.

4.2.2 Arc sine transformation consists of determining the angle (in radians) represented by a sine value. In the case of arc sine square root transformation of mortality data, the organism response proportion (proportion dead or affected; proportion surviving) is taken as the sine value, the square root of the sine value is determined, and the angle (in radians) for the square root of the sine value is determined. Whenever the response proportion is 0 or 1, a special modification of the arc sine square root transformation must be used (Bartlett, 1937). An explanation of the arc sine square root transformation and the modification is provided below.

4.2.3 Calculate the response proportion (RP) at each effluent concentration, in this case proportion surviving where:

RP = (number of surviving or unaffected organisms)/(number exposed).

Example: If 12 of 20 animals in a given treatment replicate survive:

RP = 12/20

= 0.60

4.2.4 Transform each RP to its arc sine square root, as follows:

4.2.4.1 For RPs greater than zero or less than one:

Angle (radians) = $\sqrt{RP}$

Example: If RP = 0.60:

Angle = arc sine $\sqrt{0.60}$

= arc sine 0.7746

= 0.8861 radians

4.2.4.2 Modification of the arc sine square root when RP = 0.

Angle (in radians) = arc sine $\sqrt{1/4N}$

Where: N = Number of animals/treatment replicate

Example: If 20 animals are used:

Angle = arc sine $\sqrt{1/80}$

= arc sine 0.1118

= 0.1120 radians

4.2.4.3 Modification of the arc sine square root when RP = 0

Angle = 1.5708 radians - (radians for RP = 0)

Example: Using above value:

Angle = 1.5708 - 0.1120

= 1.4588 radians

APPENDIX C

DUNNETT'S PROCEDURE

1. MANUAL CALCULATIONS

1.1 Dunnett's Procedure (Dunnett, 1955; Dunnett, 1964) is used to compare each concentration mean with the control mean to decide if any of the concentrations differ from the control. This test has an overall error rate of alpha, which accounts for the multiple comparisons with the control. It is based on the assumptions that the observations are independent and normally distributed and that the variance of the observations is homogeneous across all concentrations and control. (See Appendix B for a discussion on validating the assumptions). Dunnett's Procedure uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance. Dunnett's Procedure can only be used when the same number of replicate test vessels have been used at each concentration and the control. When this condition is not met, the *t* test with Bonferroni's adjustment is used (see Appendix D).

1.2 The data used in this example are growth data from a Mysid Survival and Growth Test, and are the same data used in Appendix B. These data are listed in Table C.1.

TABLE C.1. MYSID, *HOLMESIMYSIS COSTATA*, GROWTH DATA

		Concentration (%)		
Replicate	Control	1.80	3.20	5.60
1	0.048	0.055	0.057	0.041
2	0.058	0.048	0.050	0.040
3	0.047	0.042	0.046	0.041
4	0.058	0.041	0.043	0.043
5	0.051	0.052	0.045	0.040
Mean ($\bar{X}_i$)	0.052	0.048	0.048	0.041
Total (T_i)	0.262	0.238	0.241	0.205
i	1	2	3	4

1.3 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, as described in Table C.2:

TABLE C.2. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of effluent concentrations including the control:

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration "i"

$SST = \sum_{ij} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$SSB = \sum_i T_i^2/n_i - G^2/N$ Between Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations; $G = \sum_{i=1}^{p} T_i$

T_i = the total of the replicate measurements for concentration i

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration i

Y_{ij} = the jth observation for concentration i

1.4 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 5$

$N = 20$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 0.262$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 0.238$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} + Y_{35} = 0.241$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} + Y_{45} = 0.205$

$G = T_1 + T_2 + T_3 + T_4 = 0.946$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$$= \frac{1}{5}(0.225) - \frac{(0.946)^2}{20} = 0.000254$$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 0.0455 - \frac{(0.946)^2}{20} = 0.000754$$

$$SSW = SST - SSB = 0.000754 - 0.000254 = 0.000500$$

$S_B^2 = SSB/(p-1) = 0.000254/(4-1) = 0.0000847$

$S_W^2 = SSW/(N-p) = 0.000500/(20-4) = 0.0000313$

1.5 Summarize these data in the ANOVA table, as shown in Table C.3:

TABLE C.3. COMPLETED ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	3	0.000254	0.0000847
Within	16	0.000500	0.0000313
Total	19	0.000754	

1.6 To perform the individual comparisons, calculate the *t* statistic for each concentration and control combination, as follows:

$$t_i \; ' \; \frac{(\bar{Y}_1 \; \& \; \bar{Y}_i)}{S_w \sqrt{(1/n_1) \; \% \; (1/n_i)}}$$

Where: $\&_1$ = mean for the control

$\&_i$ = mean for each concentratioon i

S_w = square root of the within mean square

n_1 = number of replicates in the control

n_i = number of replicates for concentration i.

1.7 Table C.4 includes the calculated *t* values for each concentration and control combination.

TABLE C.4. CALCULATED *t* VALUES

Concentration (ppb)	i	t_i
1.80	2	1.131
3.20	3	1.131
5.60	4	3.111

1.8 Since the purpose of the test is only to detect a decrease in growth from the control, a one-sided test is appropriate. The critical value for the one-sided comparison is read from the table of Dunnett's "*t*" values (Table C.5; this table assumes an equal number of replicates in all treatment concentrations and the control). For this set of data, with an overall alpha level of 0.05, 16 degrees of freedom and three concentrations excluding the control, the critical value is 2.23. The mean weight for concentration "i" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Comparing each of the calculated *t* values in Table C.4 with the critical value, a significant decrease in growth from the control is detected in the 5.60% concentration. Therefore, the NOEC and the LOEC for growth are 3.20% and 5.60%, respectively.

TABLE C.5. DUNNETT'S "T" VALUES (Miller, 1981)

	(One-tailed) d^a_k																	
v k	a = .05									a = 0.1								
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
5	2.02	2.44	2.68	2.85	2.98	3.08	3.16	3.24	3.30	3.37	3.90	4.21	4.43	4.60	4.73	4.85	4.94	5.03
6	1.94	2.34	2.56	2.71	2.83	2.92	3.00	3.07	3.12	3.14	3.61	3.88	4.07	4.21	4.33	4.43	4.51	4.59
7	1.89	2.27	2.48	2.62	2.73	2.82	2.89	2.95	3.01	3.00	3.42	3.56	3.83	3.96	4.07	4.15	4.23	4.30
8	1.86	2.22	2.42	2.55	2.66	2.74	2.81	2.87	2.92	2.90	3.29	3.51	3.67	3.79	3.88	3.96	4.03	4.09
9	1.83	2.18	2.37	2.50	2.60	2.68	2.75	2.81	2.86	2.82	3.19	3.40	3.55	3.66	3.75	3.82	3.89	3.94
10	1.81	2.15	2.34	2.47	2.56	2.64	2.70	2.76	2.81	2.76	3.11	3.31	3.45	3.56	3.64	3.71	3.78	3.83
11	1.80	2.13	2.31	2.44	2.53	2.60	2.67	2.72	2.77	2.72	3.06	3.25	3.38	3.48	3.56	3.63	3.69	3.74
12	1.78	2.11	2.29	2.41	2.50	2.58	2.64	2.69	2.74	2.68	3.01	3.19	3.32	3.42	3.50	3.56	3.62	3.67
13	1.77	2.09	2.27	2.39	2.48	2.55	2.61	2.66	2.71	2.65	2.97	3.15	3.27	3.37	3.44	3.91	3.56	3.61
14	1.76	2.08	2.25	2.37	2.46	2.53	2.59	2.64	2.69	2.62	2.94	3.11	3.23	3.32	3.40	3.46	3.51	3.56
15	1.75	2.07	2.24	2.36	2.44	2.51	2.57	2.62	2.67	2.60	2.91	3.08	3.20	3.29	3.36	3.42	3.47	3.52
16	1.75	2.06	2.23	2.34	2.43	2.50	2.56	2.61	2.65	2.58	2.88	3.05	3.17	3.26	3.33	3.39	3.44	3.48
17	1.74	2.05	2.22	2.33	2.42	2.49	2.54	2.59	2.64	2.57	2.86	3.03	3.14	3.23	3.30	3.36	3.41	3.45
18	1.73	2.04	2.21	2.32	2.41	2.48	2.53	2.58	2.62	2.55	2.84	3.01	3.12	3.21	3.27	3.33	3.38	3.42
19	1.73	2.03	2.20	2.31	2.40	2.47	2.52	2.57	2.61	2.54	2.83	2.99	3.10	3.18	3.25	3.31	3.36	3.40
20	1.72	2.03	2.19	2.30	2.39	2.46	2.51	2.56	2.60	2.53	2.81	2.97	3.08	3.17	3.23	3.29	3.34	3.38
24	1.71	2.01	2.17	2.28	2.36	2.43	2.48	2.53	2.57	2.49	2.77	2.92	3.03	3.11	3.17	3.22	3.27	3.31
30	1.70	1.99	2.15	2.25	2.33	2.40	2.45	2.50	2.54	2.46	2.72	2.87	2.97	3.05	3.11	3.16	3.21	3.24
40	1.68	1.97	2.13	2.23	2.31	2.37	2.42	2.47	2.51	2.42	2.68	2.82	2.92	2.99	3.05	3.10	3.14	3.18
60	1.67	1.95	2.10	2.21	2.28	2.35	2.39	2.44	2.48	2.39	2.64	2.78	2.87	2.94	3.00	3.04	3.08	3.12
120	1.66	1.93	2.08	2.18	2.26	2.32	2.37	2.41	2.45	2.36	2.60	2.73	2.82	2.90	2.94	2.99	3.03	3.06

1.9 To quantify the sensitivity of the test, the minimum significant difference (MSD) may be calculated. The formula is as follows:

$$MSD' d\ S_w\sqrt{(1/n_1)\%(1/n)}$$

Where: d = critical value for the Dunnett's Procedure

S_w = the square root of the within mean square

n = the number of replicates at each concentration, assuming an equal number of replicates at all treatment concentrations

n_1 = number of replicates in the control

For example:

$$MSD\ '\ 2.23(0.00559)\sqrt{(1/5)\%(1/5)}$$

$$= 2.23\ (0.00559)(0.632)$$

$$= 0.00788$$

1.10 Therefore, for this set of data, the minimum difference between the control mean and a concentration mean that can be detected as statistically significant is 0.00788 mg. This represents a 15.2% reduction in mean weight from the control.

1.11 If the data have not been transformed, the MSD (and the percent decrease from the control mean that it represents) can be reported as is.

1.11.1 In the case where the data have been transformed, the MSD would be in transformed units. In this case carry out the following conversion to determine the MSD in untransformed units.

1.11.2 Subtract the MSD from the transformed control mean. Call this difference D. Next, obtain untransformed values for the control mean and the difference, D. Finally, compute the untransformed MSD as follows:

$$MSD_u = control_u - D_u$$

Where: MSD_u = the minimum significant difference for untransformed data

$Control_u$ = the untransformed control mean

D_u = the untransformed difference

1.11.3 Calculate the percent reduction from the control that MSD_u represents as:

$$\text{Percent Reduction} = \frac{MSD_u}{Control_u} \times 100$$

1.11.3.1 An example of a conversion of the MSD to untransformed units, when the arc sine square root transformation was used on the data, follows.

Step 1. Subtract the MSD from the transformed control mean. As an example, assume the data in Table C.1 were transformed by the arc sine square root transformation. Thus:

$$0.052 - 0.00788 = 0.04412$$

Step 2. Obtain untransformed values for the control mean (0.052) and the difference (0.04412) obtained in Step 1, above.

$$[\text{ Sine } (0.052)]^2 = 0.00270$$

$$[\text{ Sine } (0.04412)]^2 = 0.00195$$

Step 3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values obtained in Step 2.

$$MSD_u = 0.00270 - 0.00195 = 0.00075$$

In this case, the MSD would represent a 1.4% decrease in survival from the control [(0.00075/0.052)(100)].

2. **COMPUTER CALCULATIONS**

2.1 This computer program incorporates two analyses: an analysis of variance (ANOVA), and a multiple comparison of treatment means with the control mean (Dunnett's Procedure). The ANOVA is used to obtain the error value. Dunnett's Procedure indicates which toxicant concentration means (if any) are statistically different from the control mean at the 5% level of significance. The program also provides the minimum difference between the control and treatment means that could be detected as statistically significant, and tests the validity of the homogeneity of variance assumption by Bartlett's Test. The multiple comparison is performed based on procedures described by Dunnett (1955).

2.2 The source code for the Dunnett's program is structured into a series of subroutines, controlled by a driver routine. Each subroutine has a specific function in the Dunnett's Procedure, such as data input, transforming the data, testing for equality of variances, computing p values, and calculating the one-way analysis of variance.

2.3 The program compares up to seven toxicant concentrations against the control, and can accommodate up to 50 replicates per concentration.

2.4 If the number of replicates at each toxicant concentration and control are not equal, a *t* test with the Bonferroni adjustment is performed instead of Dunnett's Procedure (see Appendix D).

2.5 The program was written in IBM-PC FORTRAN by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled version of the program can be obtained from EMSL-Cincinnati by sending a diskette with a written request.

2.6 DATA INPUT AND OUTPUT

2.6.1 The mysid growth data from Table C.1 are used to illustrate the data input and output for this program.

2.6.2 Data Input

2.6.2.1 When the program is entered, the user is asked to select the type of data to be analyzed:

1. Response proportions, like survival or fertilization proportions data.
2. Counts and measurements, like offspring counts, cystocarp and algal cell counts, weights, chlorophyll measurements or turbidity measurements.

2.6.2.2 After the type of analysis for the data is chosen, the user has the following options:

1. Create a data file
2. Edit a data file
3. Perform analysis on existing data set
4. Stop

2.6.2.3 When Option 1 (Create a data file) is selected for response proportions, the program prompts the user for the following information:

1. Number of concentrations, including control
2. For each concentration and replicate:
   - number of organisms exposed per replicate
   - number of organisms responding per replicate (organisms surviving, replicate (organisms.)

2.6.2.4 After the data have been entered, the user may save the file on a disk, and the program returns to the main menu (see below).

2.6.2.5 Sample data input is shown in Figure C.1.

2.6.3. Program Output

2.6.3.1 When Option 3 (perform analysis on existing data set) is selected from the menu, the user is asked to select the transformation desired, and indicate whether they expect the means of the test groups to be less or greater than the mean for the control group (see Figure C.2).

```
                    EMSL Cincinnati Dunnett Software
                              Version 1.5

What type of data do you wish to analyze?

     1)  response proportions
         (like survival data or fertility proportion data)
         Note:  The program calculates a proportion after prompting for
                number of exposed organisms and number of responding
                organisms.

     2)  counts and measurements
         (like offspring counts, cystocarps and algal cell counts,
         weights, chlorophyll measurements, or turbidity measurements)


 Enter "1", "2", (or "q" to quit program): 2


Title ? Appendix C, Dunnett's Procedure Example - Mysid Data

Output to printer or disk file ? P

 1) Create a data file
 2) Edit a data file
 3) Analyze an existing data set
 4) Stop

Your choice ? 1

Number of concentrations, including control ? 4


Number of observations for conc. 1 (the control) ? 5

Enter the data for conc. 1 (the control) one observation at a time.

NO.  1? 0.048

NO.  2? 0.058

NO.  3? 0.047

NO.  4? 0.058

NO.  5? 0.051
```

Figure C.1. Sample Data Input for Dunnett's Program for Survival Data from Table C.1.

```
Enter the data for conc.  2 one observation at a time.

NO.  1? 0.055

NO.  2? 0.048

NO.  3? 0.042

NO.  4? 0.041

NO.  5? 0.052

Number of observations for conc.  3 ? 5

Enter the data for conc.  3 one observation at a time.

NO.  1? 0.057

NO.  2? 0.050

NO.  3? 0.046

NO.  4? 0.043

NO.  5? 0.045

Number of observations for conc.  4 ? 5

Enter the data for conc.  4 one observation at a time.

NO.  1? 0.041

NO.  2? 0.040

NO.  3? 0.041

NO.  4? 0.043

NO.  5? 0.040

Do you wish to save the data on disk ? Y

Disk file for output ? c:\mysid.dat
```

Figure C.1. Sample Data Input for Dunnett's Program for Survival Data from Table C.1. (Continued)

```
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 1) Create a data file
 2) Edit a data file
 3) Analyze an existing data set
 4) Stop

Your choice ? 3


File name ? c:\mysid.dat



 Available Transformations
     1)  no transform
     2)  square root
     3)  log10

Your choice ? 1



 Dunnett's test as implemented in this program is
 a one-sided test. You must specify the direction
 the test is to be run; that is, do you expect the
 means for the test concentrations to be less than
 or greater than the mean for the control
 concentration.

Direction for Dunnetts test : L=less than, G=greater than ? L
```

Figure C.2. Example of Choosing Option 3 from the Main Menu of the Dunnett Program.

2.6.3.2 Summary statistics (Figure C.3) for the raw and transformed data, if applicable, the ANOVA table, results of Bartlett's Test, the results of the multiple comparison procedure, and the minimum detectable difference are included in the program output.

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Version 1.5

Appendix C, Dunnett's Procedure Example - Mysid Data

Summary Statistics and ANOVA

Transformation = None

Conc.	n	Mean	s.d.	cv%
1 = control	5	.0524	.0053	10.2
2	5	.0476	.0061	12.8
3	5	.0482	.0055	11.5
4*	5	.0410	.0012	3.0

*) the mean for this conc. is significantly less than
the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.006974
This difference corresponds to -13.31 percent of control

Between concentrations
sum of squares = .000333 with 3 degrees of freedom.

Error mean square = .000024 with 16 degrees of freedom.

Bartlett's test p-value for equality of variances = .060

Do you wish to restart the program ?

Figure C.3. Example of Program Output for the Dunnett's Program Using the Data in Table C.1.

APPENDIX D

t TEST WITH BONFERRONI'S ADJUSTMENT

1. The *t* test with Bonferroni's adjustment is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus, Dunnett's Procedure is a more powerful test.

2. The *t* test with Bonferroni's adjustment is based on the same assumptions of normality of distribution and homogeneity of variance as Dunnett's Procedure (See Appendix B for testing these assumptions), and, like Dunnett's Procedure, uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance.

3. An example of the use of the *t* test with Bonferroni's adjustment is provided below. The data used in the example are a set of red abalone growth data. Because there are only four replicates in the highest concentration, Dunnett's Procedure cannot be used. The length data are presented in Table D.1.

TABLE D.1. GIANT KELP, *MACROCYSTIS PYRIFERA*, GROWTH DATA

	Copper Concentration (µg/L)							
Rep	Control	5.60	10.0	18.0	32.0	56.0	100.0	180.0
1	19.58	18.26	13.31	18.59	12.54	11.44	7.92	6.49
2	18.75	16.25	18.92	12.88	10.67	11.88	7.59	7.25
3	19.14	16.39	15.62	16.28	15.95	11.88	8.25	--
4	16.50	18.70	14.30	15.38	12.54	11.00	9.13	7.63
5	17.93	15.62	15.29	19.75	11.66	11.55	8.80	8.13
$\bar{X}_i$	18.38	17.04	15.49	16.58	12.67	11.55	8.34	7.38
S_i^2	1.473	1.827	4.498	7.327	3.953	0.133	0.396	0.478
i	1	2	3	4	5	6	7	8

3.1 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, as described in Table D.2:

TABLE D.2. ANOVA TABLE

Source	*df*	*Sum of Squares (SS)*	*Mean Square(MS) (SS/df)*
Between	*p - 1*	*SSB*	$S_B^2 = SSB/(p-1)$
Within	*N - p*	*SSW*	$S_W^2 = SSW/(N-p)$

Where: p = number of effluent concentrations including the control

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration i

$$SST = \sum_{ij} Y_{ij}^2 - G^2/N$$

Total Sum of Squares

$$SSB = \sum_i T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

Where: G = The grand total of all sample observations; $G = \sum_{i=1}^{p} T_i$

T_i = The total of the replicate measurements for concentration i

Y_{ij} = The jth observation for concentration i

3.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = n_7 = 5; \quad n_8 = 4$

$N = 39$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 91.90$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 85.22$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} + Y_{35} = 77.44$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} + Y_{45} = 82.88$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} + Y_{55} = 63.36$
$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} + Y_{65} = 57.75$
$T_7 = Y_{71} + Y_{72} + Y_{73} + Y_{74} + Y_{75} = 41.69$
$T_8 = Y_{81} + Y_{82} + Y_{83} + Y_{84} = 29.50$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 + T_7 + T_8 = 529.74$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$$= 7749.905 - \frac{(529.74)^2}{39} = 554.406$$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 7829.764 - \frac{(529.74)^2}{39} = 634.265$$

$$SSW = SST - SSB = 634.265 - 554.406 = 79.859$$

$S_B^2 = SSB/(p-1) = 554.406/(8-1) = 79.201$

$S_W^2 = SSW/(N-p) = 79.859/(39-8) = 2.576$

3.3 Summarize these calculations in the ANOVA table (Table D.3):

TABLE D.3. COMPLETED ANOVA TABLE FOR THE *t* TEST WITH BONFERRONI'S ADJUSTMENT EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	7	554.406	79.201
Within	31	79.859	2.576
Total	38	634.265	

3.4 To perform the individual comparisons, calculate the *t* statistic for each concentration and control combination, as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean for concentration i

$\bar{Y}_1$ = mean for the control

S_w = square root of the within mean square

n_1 = number of replicates in the control.

n_i = number of replicates for concentration i.

3.5 Table D.4 includes the calculated *t* values for each concentration and control combination.

TABLE D.4. CALCULATED t VALUES

Concentration (µg/L)	i	t_i
5.6	2	1.320
10.0	3	2.847
18.0	4	1.773
32.0	5	5.625
56.0	6	6.728
100.0	7	9.891
180.0	8	10.217

3.6 Since the purpose of this test is to detect a significant reduction in mean length, a one-sided test is appropriate. The critical value for this one-sided test is found in Table D.5. For an overall alpha level of 0.05, 31 degrees of freedom for error and seven concentrations (excluding the control) the approximate critical value is 2.597. The mean length for concentration "i" is considered significantly less than the mean length for the control if t_i is greater than the critical value. Comparing each of the calculated t values in Table D.4 with the critical value, the 10.0 µg/L, 32 µg/L, 56.0 µg/L, 100.0 µg/L, 180.0 µg/L concentrations have significantly lower mean length than the control. Because the 10.0 µg/L concentration shows signigicantly lower mean length that the control while the higher 18.0 µg/L concentration does not, these test results are considered to have an anomalous dose-response relationship and it is recommended that the test be repeated. If an NOEC and LOEC must be determined for this test, the lowest concentration with significant growth impairment versus the control is considered to the LOEC for growth. Thus, for this test, the NOEC and LOEC would be 5.6 µg/L and 10.0 µg/L, respectively.

TABLE D.5. CRITICAL VALUES FOR "t" FOR THE t TEST WITH BONFERRONI'S ADJUSTMENT P = 0.05
CRITICAL LEVEL, ONE TAILED

d.f.	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
1	6.314	12.707	19.002	25.452	31.821	38.189	44.556	50.924	57.290	63.657
2	2.920	4.303	5.340	6.206	6.965	7.649	8.277	8.861	9.408	9.925
3	2.354	3.183	3.741	4.177	4.541	4.857	5.138	5.392	5.626	5.841
4	2.132	2.777	3.187	3.496	3.747	3.961	4.148	4.315	4.466	4.605
5	2.016	2.571	2.912	3.164	3.365	3.535	3.681	3.811	3.927	4.033
6	1.944	2.447	2.750	2.969	3.143	3.288	3.412	3.522	3.619	3.708
7	1.895	2.365	2.642	2.842	2.998	3.128	3.239	3.336	3.422	3.500
8	1.860	2.307	2.567	2.752	2.897	3.016	3.118	3.206	3.285	3.356
9	1.834	2.263	2.510	2.686	2.822	2.934	3.029	3.111	3.185	3.250
10	1.813	2.229	2.406	2.634	2.764	2.871	2.961	3.039	3.108	3.170
11	1.796	2.201	2.432	2.594	2.719	2.821	2.907	2.981	3.047	3.106
12	1.783	2.179	2.404	2.561	2.681	2.778	2.863	2.935	2.998	3.055
13	1.771	2.161	2.380	2.533	2.651	2.746	2.827	2.897	2.958	3.013
14	1.762	2.145	2.360	2.510	2.625	2.718	2.797	2.864	2.924	2.977
15	1.754	2.132	2.343	2.490	2.603	2.694	2.771	2.837	2.895	2.947
16	1.746	2.120	2.329	2.473	2.584	2.674	2.749	2.814	2.871	2.921
17	1.740	2.110	2.316	2.459	2.567	2.655	2.729	2.793	2.849	2.899
18	1.735	2.101	2.305	2.446	2.553	2.640	2.712	2.775	2.830	2.879
19	1.730	2.094	2.295	2.434	2.540	2.626	2.697	2.759	2.813	2.861
20	1.725	2.086	2.206	2.424	2.528	2.613	2.684	2.745	2.798	2.846
21	1.721	2.080	2.278	2.414	2.518	2.602	2.672	2.732	2.785	2.832
22	1.718	2.074	2.271	2.406	2.509	2.592	2.661	2.721	2.773	2.819
23	1.714	2.069	2.264	2.398	2.500	2.583	2.651	2.710	2.762	2.808
24	1.711	2.064	2.258	2.391	2.493	2.574	2.642	2.701	2.752	2.797
25	1.709	2.060	2.253	2.385	2.486	2.566	2.634	2.692	2.743	2.788

TABLE D.5. CRITICAL VALUES FOR "t" FOR THE t TEST WITH BONFERRONI'S ADJUSTMENT
P = 0.05 CRITICAL LEVEL, ONE TAILED (CONTINUED)

df	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
29	1.700	2.046	2.235	2.364	2.463	2.541	2.607	2.664	2.713	2.757
30	1.698	2.043	2.231	2.360	2.458	2.536	2.602	2.658	2.707	2.750
31	1.696	2.040	2.228	2.356	2.453	2.531	2.597	2.652	2.701	2.745
32	1.694	2.037	2.224	2.352	2.449	2.527	2.592	2.647	2.696	2.739
33	1.693	2.035	2.221	2.349	2.445	2.523	2.587	2.643	2.691	2.734
34	1.691	2.033	2.219	2.346	2.442	2.519	2.583	2.638	2.686	2.729
35	1.690	2.031	2.216	2.342	2.438	2.515	2.579	2.634	2.682	2.724
36	1.689	2.029	2.213	2.340	2.435	2.512	2.575	2.630	2.678	2.720
37	1.688	2.027	2.211	2.337	2.432	2.508	2.572	2.626	2.674	2.716
38	1.686	2.025	2.209	2.334	2.429	2.505	2.568	2.623	2.670	2.712
39	1.685	2.023	2.207	2.332	2.426	2.502	2.565	2.619	2.667	2.708
40	1.684	2.022	2.205	2.329	2.424	2.499	2.562	2.616	2.663	2.705
50	1.676	2.009	2.189	2.311	2.404	2.478	2.539	2.592	2.638	2.678
60	1.671	2.001	2.179	2.300	2.391	2.463	2.324	2.576	2.621	2.661
70	1.667	1.995	2.171	2.291	2.381	2.453	2.513	2.564	2.609	2.648
80	1.665	1.991	2.166	2.285	2.374	2.446	2.505	2.556	2.600	2.639
90	1.662	1.987	2.162	2.280	2.369	2.440	2.499	2.549	2.593	2.632
100	1.661	1.984	2.158	2.276	2.365	2.435	2.494	2.544	2.588	2.626
110	1.659	1.982	2.156	2.273	2.361	2.432	2.490	2.540	2.583	2.622
120	1.658	1.980	2.153	2.270	2.358	2.429	2.487	2.536	2.580	2.618
Infinite	1.645	1.960	2.129	2.242	2.327	2.394	2.450	2.498	2.540	2.576

d.f. = Degrees of freedom for MSE (Mean Square Error) from ANOVA.
K = Number of concentrations to be compared to the control.

APPENDIX E

STEEL'S MANY-ONE RANK TEST

1. Steel's Many-one Rank Test is a nonparametric test for comparing treatments with a control. This test is an alternative to Dunnett's Procedure, and may be applied to data when the normality assumption has not been met. Steel's Test requires equal variances across the treatments and the control, but it is thought to be fairly insensitive to deviations from this condition (Steel, 1959). The tables for Steel's Test require an equal number of replicates at each concentration. If this is not the case, use Wilcoxon's Rank Sum Test, with Bonferroni's adjustment (See Appendix F).

2. For an analysis using Steel's Test, for each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to the observation. (Extensive ties would invalidate this procedure). The sum of the ranks within each concentration is then calculated. To determine if the response in a concentration is significantly less than the response in the control, the rank sum for each concentration is compared to the significant values of rank sums given later in the section. In this table, k equals the number of treatments excluding the control and n equals the number of replicates for each concentration and the control.

3. An example of the use of this test is provided below. The test employs embryo-larval development data from a bivalve 48-hour chronic test. The data are listed in Table E.1.

4. For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, 3, ..., 8) to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to each tied observation.

5. An example of assigning ranks to the combined data for the control and 0.13 µg/L copper concentration is given in Table E.2.

This ranking procedure is repeated for each control and concentration combination. The complete set of rankings is listed in Table E.3. The ranks are then summed for each toxicant concentration, as shown in Table E.4.

6. For this set of data, determine if the development in any of the effluent concentrations is significantly lower than the development of the control organisms. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for the development at each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the survival would be considered to be significantly lower than the control. At a probability level of 0.05, the critical rank sum in a test with five concentrations and four replicates per concentration, is 10 (see Table F.4).

7. Since the rank sums for the 0.50 µg/L and 1.00 µg/L concentration levels are equal to the critical value, the proportions of normal development in those concentrations are considered significantly less than that in the control. Since no other rank sum is less than or equal to the critical value, no other concentration has a significantly lower proportion normal than the control. Because the 0.50 µg/L concentration shows signigicantly lower normal development than the control while the higher 2.00 µg/L concentration does not, these test results are considered to have an anomalous dose-response relationship and it is recommended that the test be repeated. If an NOEC and LOEC must be determined for this test, the lowest concentration with significant impairment versus the control is considered to the LOEC for growth. Thus, for this test, the NOEC and LOEC would be 0.25 µg/L and 0.50 µg/L, respectively.

TABLE E.1. BIVALVE EMBRYO-LARVAL DEVELOPMENT DATA

		Copper Concentration (µg/L)					
	Replicate	Control	0.13	0.25	0.50	1.00	2.00
	A	1.00	0.96	0.92	0.91	0.88	1.00
RAW	B	0.96	0.97	0.95	0.93	0.83	0.67
	C	1.00	1.00	0.90	0.88	0.88	0.75
	D	0.97	0.96	0.96	0.93	0.82	0.60
ARC SINE	A	1.571	1.369	1.284	1.266	1.217	1.571
SQUARE ROOT	B	1.369	1.397	1.345	1.303	1.146	0.959
TRANSFORMED	C	1.571	1.571	1.249	1.217	1.217	1.047
	D	1.397	1.369	1.369	1.303	1.133	0.886
Mean ($\bar{x}_i$)		1.477	1.427	1.312	1.272	1.178	1.116
S_i^2		0.01191	0.00945	0.00303	0.00166	0.00203	0.09644
i		1	2	3	4	5	6

TABLE E.2. ASSIGNING RANKS TO THE CONTROL AND 0.13 µg/L CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Normal	Concentration
2	1.369	0.13 µg/L
2	1.369	0.13 µg/L
2	1.369	Control
4.5	1.397	0.13 µg/L
4.5	1.397	Control
7	1.571	0.13 µg/L
7	1.571	Control
7	1.571	Control

TABLE E.3. TABLE OF RANKS[1]

Replicate	Control	Copper Concentration (µg/L) 0.13	0.25
1	1.571(7,7.5,7.5,7.5,7)	1.369(2)	1.284(2)
2	1.369(2,4.5,5,5,4)	1.397(4.5)	1.345(3)
3	1.571(7,7.5,7.5,7.5,7)	1.571(7)	1.249(1)
4	1.397(4.5,6,6,6,5)	1.369(2)	1.369(4.5)

Replicate	Copper Concentration (µg/L) (Continued) 0.50	1.00	2.00
1	1.266(2)	1.217(3.5)	1.571(7)
2	1.303(3.5)	1.146(2)	0.959(2)
3	1.217(1)	1.217(3.5)	1.047(3)
4	1.303(3.5)	1.133(1)	0.886(1)

[1]Control ranks are given in the order of the concentration with which they were ranked.

TABLE E.4. RANK SUMS

Concentration µg/L Copper)	Rank Sum
0.13	15.5
0.25	10.5
0.50	10.0
1.00	10.0
2.00	13.0

TABLE E.5. SIGNIFICANT VALUES OF RANK SUMS: JOINT CONFIDENCE COEFFICIENTS OF 0.95 (UPPER) and 0.99 (LOWER) FOR ONE-SIDED ALTERNATIVES (Steel, 1959)

		k = number of treatments (excluding control)							
n	%	2	3	4	5	6	7	8	9
	%								
	%								
4	%	11	10	10	10	10	--	--	--
	%	--	--	--	--	--	--	--	--
5	%	18	17	17	16	16	16	16	15
	%	15	--	--	--	--	--	--	--
6	%	27	26	25	25	24	24	24	23
	%	23	22	21	21	--	--	--	--
7	%	37	36	35	35	34	34	33	33
	%	32	31	30	30	29	29	29	29
8	%	49	48	47	46	46	45	45	44
	%	43	42	41	40	40	40	39	39
9	%	63	62	61	60	59	59	58	58
	%	56	55	54	53	52	52	51	51
10	%	79	77	76	75	74	74	73	72
	%	71	69	68	67	66	66	65	65
11	%	97	95	93	92	91	90	90	89
	%	87	85	84	83	82	81	81	80
12	%	116	114	112	111	110	109	108	108
	%	105	103	102	100	99	99	98	98
13	%	138	135	133	132	130	129	129	128
	%	125	123	121	120	119	118	117	117
14	%	161	158	155	154	153	152	151	150
	%	147	144	142	141	140	139	138	137
15	%	186	182	180	178	177	176	175	174
	%	170	167	165	164	162	161	160	160
16	%	213	209	206	204	203	201	200	199
	%	196	192	190	188	187	186	185	184
17	%	241	237	234	232	231	229	228	227
	%	223	219	217	215	213	212	211	210
18	%	272	267	264	262	260	259	257	256
	%	252	248	245	243	241	240	239	238
19	%	304	299	296	294	292	290	288	287
	%	282	278	275	273	271	270	268	267
20	%	339	333	330	327	325	323	322	320
	%	315	310	307	305	303	301	300	299

APPENDIX F

WILCOXON RANK SUM TEST

1. Wilcoxon's Rank Sum Test is a nonparametric test, to be used as an alternative to Steel's Many-one Rank Test when the number of replicates are not the same at each concentration. A Bonferroni's adjustment of the pairwise error rate for comparison of each concentration versus the control is used to set an upper bound of alpha on the overall error rate, in contrast to Steel's Many-one Rank Test, for which the overall error rate is fixed at alpha. Thus, Steel's Test is a more powerful test.

2. The use of this test may be illustrated with devlopment data from the red abalone test in Table F.1. The control group has four replicates while each of the concentration levels has five replicates. Since there is 100% abnormality in all replicates for the 5.6% and 10.0% concentrations, they are not included in the statistical analysis and are considered qualitative abnormality effects.

3. For each concentration and control combination, combine the data and arrange the values in order of size, from smallest to largest. Assign ranks to the ordered observations (a rank of 1 to the smallest, 2 to the next smallest, etc.). If ties in rank occur, assign the average rank to each tied observation.

4. An example of assigning ranks to the combined data for the control and effluent concentration 0.56% is given in Table F.2. This ranking procedure is repeated for each of the three remaining control versus test concentration combinations. The complete set of ranks is listed in Table F.3. The ranks are then summed for each effluent concentration, as shown in Table F.4.

5. For this set of data, determine if the development in any of the test concentrations is significantly lower than the development in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for fecundity of each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the fecundity would be considered to be significantly lower than the control. At a

probability level of 0.05, the critical rank in a test with four concentrations (excluding the control), four control replicates, and five concentration replicates is 15 (see Table F.5, for K = 4).

TABLE F.1. RED ABALONE, HALIOTUS RUFESCENS, SHELL DEVELOPMENT DATA

			Effluent Concentration (%)					
	Replicate	Dilution Control	0.56	1.00	1.80	3.20	5.6	10.0
RAW	A	0.99	0.99	0.99	0.99	0.39	0	0
	B	0.99	0.99	1.00	0.99	0.57	0	0
	C	0.99	0.98	0.99	0.99	0.61	0	0
	D	1.00	1.00	0.99	0.98	0.65	0	0
	E		1.00	1.00	0.97	0.80	0	0
ARC SINE SQUARE ROOT TRANSFORMED	A	1.471	1.471	1.471	1.471	0.674	-	-
	B	1.471	1.471	1.521	1.471	0.856	-	-
	C	1.471	1.429	1.471	1.471	0.896	-	-
	D	1.521	1.521	1.471	1.429	0.938	-	-
	E		1.521	1.521	1.397	1.107	-	-
Mean ($\bar{x}_i$)		1.484	1.483	1.491	1.448	0.894	-	-
S_i^2		0.000625	0.001523	0.000750	0.001137	0.024288	-	-
i		1	2	3	4	5	6	7

TABLE F.2. ASSIGNING RANKS TO THE CONTROL AND 0.56% CONCENTRATION LEVEL FOR THE WILCOXON RANK SUM TEST WITH THE BONFERRONI ADJUSTMENT

Rank	Transformed Proportion Normal	Concentration
1	1.429	0.56 %
4	1.471	0.56 %
4	1.471	0.56 %
4	1.471	Control
4	1.471	Control
4	1.471	Control
8	1.521	0.56 %
8	1.521	0.56 %
8	1.521	Control

TABLE F.3. TABLE OF RANKS[1]

Replicate	Control	Effluent Concentration (%) 0.56	1.00	1.80	3.20
1	1.471(4,3.5,5.5,7)	1.471(4)	1.471(3.5)	1.471(5.5)	0.674(1)
2	1.471(4,3.5,5.5,7)	1.471(4)	1.521(8)	1.471(5.5)	0.856(2)
3	1.471(4,3.5,5.5,7)	1.429(1)	1.471(3.5)	1.471(5.5)	0.896(3)
4	1.521(8,8,9,9)	1.521(8)	1.471(3.5)	1.429(2)	0.938(4)
5		1.521(8)	1.521(8)	1.397(1)	1.107(5)

[1]Control ranks are given in the order of the concentration with which they were ranked.

6. Comparing the rank sums in Table F.4 to the appropriate critical rank, the rank sum for the 3.20% concentration level is equal to the critical value, so the proportion normal in that concentration is considered significantly less than that in the control. Since no other rank sum is less than or equal to the critical value, no other concentration has a significantly lower proportion normal than the control. Hence, the NOEC and the LOEC are 1.80% and 3.20%, respectively.

TABLE F.4. RANK SUMS

Concentration (% Effluent)	*Rank Sum*
0.56	*25.0*
1.00	*26.5*
1.80	*19.5*
3.20	*15.0*

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
1	3	6	10	16	23	30	39	49	59
	4	6	11	17	24	32	41	51	62
	5	7	12	19	26	34	44	54	66
	6	8	13	20	28	36	46	57	69
	7	8	14	21	29	39	49	60	72
	8	9	15	23	31	41	51	63	72
	9	10	16	24	33	43	54	66	79
	10	10	17	26	35	45	56	69	82
2	3	--	--	15	22	29	38	47	58
	4	--	10	16	23	31	40	49	60
	5	6	11	17	24	33	42	52	63
	6	7	12	18	26	34	44	55	66
	7	7	13	20	27	36	46	57	69
	8	8	14	21	29	38	49	60	72
	9	8	14	22	31	40	51	62	75
	10	9	15	23	32	42	53	65	78
3	3	--	--	--	21	29	37	46	57
	4	--	10	16	22	30	39	48	59
	5	--	11	17	24	32	41	51	62
	6	6	11	18	25	33	43	53	65
	7	7	12	19	26	35	45	56	68
	8	7	13	20	28	37	47	58	70
	9	7	13	21	29	39	49	61	73
	10	8	14	22	31	41	51	63	76
4	3	--	--	--	21	28	37	46	56
	4	--	--	15	22	30	38	48	59
	5	--	10	16	23	31	40	50	61
	6	6	11	17	24	33	42	52	64
	7	6	12	18	26	34	44	55	67
	8	7	12	19	27	36	46	57	69
	9	7	13	20	28	38	48	60	72
	10	7	14	21	30	40	50	62	75

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
5	3	--	--	--	--	28	36	46	56
	4	--	--	15	22	29	38	48	58
	5	--	10	16	23	31	40	50	61
	6	--	11	17	24	32	42	52	63
	7	6	11	18	25	34	43	54	66
	8	6	12	19	27	35	45	56	68
	9	7	13	20	28	37	47	59	71
	10	7	13	21	29	39	49	61	74
6	3	--	--	--	--	28	36	45	56
	4	--	--	15	21	29	38	47	58
	5	--	10	16	22	30	39	49	60
	6	--	11	16	24	32	41	51	63
	7	6	11	17	25	33	43	54	65
	8	6	12	18	26	35	45	56	68
	9	6	12	19	27	37	47	58	70
	10	7	13	20	29	38	49	60	73
7	3	--	--	--	--	--	36	45	56
	4	--	--	--	21	29	37	47	58
	5	--	--	15	22	30	39	49	60
	6	--	10	16	23	32	41	51	62
	7	--	11	17	25	33	43	53	65
	8	6	11	18	26	35	44	55	67
	9	6	12	19	27	36	46	58	70
	10	7	13	20	28	38	48	60	72
8	3	--	--	--	--	--	36	45	55
	4	--	--	--	21	29	37	47	57
	5	--	--	15	22	30	39	49	59
	6	--	10	16	23	31	40	51	62
	7	--	11	17	24	33	42	53	64
	8	6	11	18	25	34	44	55	67
	9	6	12	19	27	36	46	57	69
	10	6	12	19	28	37	48	59	72

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
9	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	30	39	48	59
	6	--	10	16	23	31	40	50	62
	7	--	10	17	24	33	42	52	64
	8	--	11	18	25	34	44	55	66
	9	6	11	18	26	35	46	57	69
	10	6	12	19	28	37	47	59	71
10	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	29	38	48	59
	6	--	10	16	23	31	40	50	61
	7	--	10	16	24	32	42	52	64
	8	--	11	17	25	34	43	54	66
	9	6	11	18	26	35	45	56	68
	10	6	12	19	27	37	47	58	71

APPENDIX G

SINGLE CONCENTRATION TOXICITY TEST - COMPARISON OF CONTROL WITH 100% EFFLUENT OR RECEIVING WATER OR COMPARISON OF DILUTION AND BRINE CONTROLS

1. To statistically compare a control with one concentration, such as 100% effluent or the instream waste concentration, a *t* test is the recommended analysis. The *t* test is based on the assumptions that the observations are independent and normally distributed and that the variances of the observations are equal between the two groups.

2. Shapiro-Wilk's test may be used to test the normality assumption (See Appendix B for details). For the two sample case, the datasets must be tested for normality separately. If either set of data does not meet the normality assumption, the nonparametric test, Wilcoxon's Rank Sum Test, may be used to analyze the data. An example of this test is given in Appendix F. Since a control and one concentration are being compared, the K = 1 section of Table F.5 contains the needed critical values for one-sided tests. An additional reference, such as Snedecor and Cochran (1980) must be used to determine critical values for two-sided tests, such as comparing brine and dilution controls.

3. The *F* test for equality of variances is used to test the homogeneity of variance assumption. When conducting the *F* test, the alternative hypothesis of interest is that the variances are not equal.

4. To make the two-tailed *F* test at the 0.01 level of significance, put the larger of the two variances in the numerator of *F*.

$$F \text{ '} \frac{S_1^2}{S_2^2} \quad \text{where } S_1^2 > S_2^2$$

5. Compare *F* with the 0.005 level of a tabled *F* value with n_1 - 1 and n_2 - 1 degrees of freedom, where n_1 and n_2 are the number of replicates for each of the two groups.

6. A set of mysid growth data from a single-concentration effluent test will be used to illustrate the *F* test. The raw data, mean and variance for the two controls are given in Table G.1. The data from each concentration meets the assumption of normality.

TABLE G.1. MYSID, HOLMESIMYSIS COSTATA, GROWTH DATA FROM A SINGLE-CONCENTATION EFFLUENT TEST

	Replicate	Control	Effluent
RAW	A	0.048	0.041
	B	0.058	0.033
	C	0.047	0.044
	D	0.055	0.040
	E	0.051	0.043
Mean ($\bar{X}_i$)		0.052	0.040
S_i^2		0.0000217	0.0000187
i		1	2

7. Since the variability of the control is greater than the variability of the effluent concentration, S^2 for the control is placed in the numerator of the F statistic and S^2 for the effluent concentration control is placed in the denominator.

$$F = \frac{0.0000217}{0.0000187} = 1.160$$

8. There are 5 replicates for the each groups, so the numerator and denominator degrees of freedom, n_i - 1, are both 4. For a two-tailed test at the 0.01 level of significance, the critical F value is obtained from a table of the *F* distribution (Snedecor and Cochran, 1980). The critical *F* value for this test is 23.16. Since 2.41 is not greater than 23.16, conclude that the variances of the brine and dilution controls are homogeneous.

9. Equal Variance *t* Test.

9.1 To perform the *t* test, calculate the following test statistic:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{S_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where: $\bar{Y}_1$ = mean for the control

$\bar{Y}_2$ = mean for the effluent concentration

$$S_p = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}$$

S_1^2 = estimate of the variance for the control

S_2^2 = estimate of the variance for the effluent concentration

n_1 = number of replicates for the control

n_2 = number of replicates for the effluent concentration

9.2 Since we are concerned here with a decrease in response from the control, a one-tailed test is appropriate. Thus, we will compare the calculated *t* with a critical *t*, where the critical *t* is at the 5% level of significance with $n_1 + n_2 - 2$ degrees of freedom. If the calculated *t* exceeds the critical *t*, the mean responses are declared different.

9.3 When comparing brine and dilution controls, the concern is for any difference between the two control groups, and a two-tailed test is appropriate. In that case, the calculated *t* would be compared with a critical *t*, where the critical *t* is a two-tailed value at the 5% level of significance with $n_1 + n_2 - 2$ degrees of freedom. If the absolute value of the calculated *t* exceeds the critical *t*, the mean responses are declared different.

9.4 Using the data from Table G.1 to illustrate the *t* test, the calculation of *t* is as follows:

$$t = \frac{0.052 - 0.040}{0.00449\sqrt{\frac{1}{5} + \frac{1}{5}}} = 4.226$$

$$S_p = \sqrt{\frac{(5-1)0.0000217 + (5-1)0.0000187}{5 + 5 - 2}} = 0.00449$$

Where:

9.5 For a one-tailed test at the 0.05 level of significance and 8 degrees of freedom, the appropriate critical *t* value is 1.860. Note: Table D.5 for K = 1 includes the critical *t* values for comparing two groups in a one-tailed test. Since t = 4.226 is greater than 1.860, conclude that the growth in the effluent concentration is significantly less than the control group growth.

9.6 Critical *t* values for two-tailed tests, such as those needed in comparing a brine control and a dilution control, can be found in a table of the *t* distribution, such as the one in Snedecor and Cochran, 1980. Note that the critical *t* for a two-tailed test is the upper-tail value at the a/2 level of significance.

10. UNEQUAL VARIANCE *t* TEST.

10.1 If the *F* test for equality of variance fails, the *t* test is still a valid test. However, the denominator of the *t* statistic is adjusted as follows:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where: $\bar{Y}_1$ = mean for the control

$\bar{X}_2$ = mean for the effluent concentration

S_1^2 = estimate of the variance for the control

S_2^2 = estimate of the variance for the effluent concentration

n_1 = number of replicates for the control

n_2 = number of replicates for the effluent concentration

10.2 Additionally, the degrees of freedom for the test are adjusted using the following formula:

$$df = \frac{(n_1 - 1)(n_2 - 1)}{(n_2 - 1)C^2 + (1 - C)^2(n_1 - 1)}$$

Where:

$$C = \frac{\frac{S_1^2}{n_1}}{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

10.3 The modified degrees of freedom is usually not an integer. Common practice is to round down to the nearest integer.

10.4 The *t* test is then conducted as the equal variance *t* test. The calculated *t* is compared to the critical *t* at the 0.05 significance level with the modified degrees of freedom. If the calculated *t* exceeds the critical *t*, the mean responses are found to be statistically different.

APPENDIX H

PROBIT ANALYSIS

1. This program calculates the EC1 and EC50 (or LC1 and LC50), and the associated 95% confidence intervals.

2. The program is written in IBM PC Basic for the IBM compatible PC by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled, executable version of the program and supporting documentation can be obtained from EMSL-Cincinnati by sending a written request to EMSL at 3411 Church Street, Cincinnati, OH 45244.

2.1 A set of mortality data from a mysid survival and growth test is given in Table H.1. The program's data input routine is illustrated with this data in Figure H.1. The program begins with a request for the following information:

1. Desired output of abbreviated (A) or full (F) output? (Note: only abbreviated output is shown below.)
2. Output designation (P = printer, D = disk file).
3. Title for the output.
4. The number of exposure concentrations.
5. Toxicant concentration data.2.2 The program output for the abbreviated output options, shown in Figure H.2, includes the following:

TABLE H.1. DATA FOR PROBIT ANALYSIS

		Concentration (%)				
	Control	1.80	3.20	5.60	10.0	18.0
No. Dead	1	0	3	9	24	25
No. Exposed	25	25	25	25	25	25

2.2 The program output for the abbreviated output options, shown in figure H.2, includes the following:

1. A table of the observed proportion responding and the proportion responding adjusted for the controls.
2. The calculated chi-square statistic for heterogeneity and the tabular value. This test is one indicator of how well the data fit the model. The program will issue a warning when the test indicates that the data do not fit the model.
3. The estimated LC1 and LC50 values and associated 95% confidence intervals.

```
$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$
$                                                             $
$                 EPA PROBIT ANALYSIS PROGRAM                 $
$              USED FOR CALCULATING LC/EC VALUES              $
$                         Version 1.5                         $
$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$
```

Do you wish abbreviated (A) or full (F) input/output? A
Output to printer (P) or disk file (D)? P
Title ? Example of Probit Analysis for Appendix H

Number responding in the control group = ? 1
Number of animals exposed in the concurrent control group = ? 25
Number of exposure concentrations, exclusive of controls ? 5

Input data starting with the lowest exposure concentration

Concentration = ? 1.80
Number responding = ? 0
Number exposed = ? 25

Concentration = ? 3.20
Number responding = ? 3
Number exposed = ? 25

Concentration = ? 5.60
Number responding = ? 9
Number exposed = ? 25

Concentration = ? 10.0
Number responding = ? 24
Number exposed = ? 25

Concentration = ? 18.0
Number responding = ? 25
Number exposed = ? 25

Number	Conc.	Number Resp.	Number Exposed
1	1.8000	0	25
2	3.2000	3	25
3	5.6000	9	25
4	10.0000	24	25
5	18.0000	25	25

Do you wish to modify your data ? N

The number of control animals which responded = 1
The number of control animals exposed = 25
Do you wish to modify these values ? N

Example of Probit Analysis for Appendix H

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	25	1	0.0400	0.0000
1.8000	25	0	0.0000	-.0306
3.2000	25	3	0.1200	0.0930
5.6000	25	9	0.3600	0.3404
10.0000	25	24	0.9600	0.9588
18.0000	25	25	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 3.004
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 7.815

Example of Probit Analysis for Appendix H

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	95% Confidence Limits Lower	Upper
LC/EC 1.00	2.642	1.384	3.519
LC/EC 50.00	5.973	4.998	6.920

Figure H.2. USEPA Probit Analysis Program used for Calculating LC/EC Values, Version 1.5.

APPENDIX I

SPEARMAN-KARBER METHOD

1. The Spearman-Karber Method is a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978). The Spearman-Karber Method estimates the mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Spearman-Karber Method is recommended when partial mortalities occur in the test solutions, but the data do not fit the Probit model.

4. To calculate the LC50 using the Spearman-Karber Method, the following must be true: 1) the smoothed adjusted proportion mortality for the lowest effluent concentration (not including the control) must be zero, and 2) the smoothed adjusted proportion mortality for the highest effluent concentration must be one.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed adjusted proportion mortalities must be between zero and one.

6. The Spearman-Karber Method is illustrated below using a set of mortality data from a Mysid Survival and Growth test. These data are listed in Table I.1.

TABLE I.1. EXAMPLE OF SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A MYSID SURVIVAL AND GROWTH TEST (25 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	*Number of Mortalities*	*Mortality Proportion*
Control	*2*	*0.08*
6.25	*2*	*0.08*
12.5	*0*	*0.00*
25.0	*3*	*0.12*
50.0	*16*	*0.64*
100.0	*25*	*1.00*

7. Let p_0, p_1, ..., p_k denote the observed response proportion mortalities for the control and k effluent concentrations. The first step is to smooth the p_i if they do not satisfy p_0 # p_1 # ... # p_k. The smoothing process replaces any adjacent p_i's that do not conform to p_0 # p_1 # ... # p_k with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i\&1}^{s} \text{ ' } p_i^{s} \text{ ' } (p_i\%p_{i\&1})/2$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

7.1 For the data in this example, because the observed mortality proportions for the control and the 6.25% effluent concentration are greater than the observed response proportions for the 12.5% effluent concentration, the responses for these three groups must be averaged:

$$p_0^s \text{ ' } p_1^s \text{ ' } p_2^s \text{ ' } \frac{0.08\%0.08\%0.00}{3} \text{ ' } \frac{0.16}{3} \text{ ' } 0.053$$

7.2 Since $p_3 = 0.12$ is larger than p_2^s, set $p_3^s = 0.12$. Similarly, $p_4 = 0.64$ is larger than p_3^s, so set $p_4^s = 0.64$. Finally, $p_5 = 1.00$ is larger than p_4^s, so set $p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table I.2.

TABLE I.2. EXAMPLE OF SPEARMAN-KARBER METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A MYSID SURVIVAL AND GROWTH TEST

Effluent Concentration %	*Mortality Proportion*	*Smoothed Mortality Proportion*	*Smoothed, Adjusted Mortality Proportion*
Control	*0.08*	*0.053*	*0.000*
6.25	*0.08*	*0.053*	*0.000*
12.5	*0.00*	*0.053*	*0.000*
25.0	*0.12*	*0.120*	*0.071*
50.0	*0.64*	*0.640*	*0.620*
100.0	*1.00*	*1.000*	*1.000*

8. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

8.1 For the data in this example, the data for each effluent concentration must be adjusted for control mortality using Abbott's formula, as follows:

$$p_0^a = p_1^a = p_2^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.053 - 0.053}{1 - 0.053} = \frac{0.0}{0.947} = 0.0$$

$$p_3^a = \frac{p_3^s - p_0^s}{1 - p_0^s} = \frac{0.120 - 0.053}{1 - 0.053} = \frac{0.067}{0.947} = 0.071$$

$$p_4^a = \frac{p_4^s - p_0^s}{1 - p_0^s} = \frac{0.640 - 0.053}{1 - 0.053} = \frac{0.587}{0.947} = 0.620$$

$$p_5^a = \frac{p_5^s - p_0^s}{1 - p_0^s} = \frac{1.000 - 0.053}{1 - 0.053} = \frac{0.947}{0.947} = 1.000$$

The smoothed, adjusted response proportions for the effluent concentrations are shown in Table I.2. A plot of the smoothed, adjusted data is shown in Figure I.1.

9. Calculate the $\log_{10}$ of the estimated LC50, m, as follows:

$$m = \sum_{i=1}^{k-1} \frac{(p_{i+1}^a - p_i^a)(X_i + X_{i+1})}{2}$$

Where: p_i^a = the smoothed adjusted proportion mortality at concentration i

X_i = the $\log_{10}$ of concentration i

k = the number of effluent concentrations tested, not including the control.

9.1 For this example, the $\log_{10}$ of the estimated LC50, m, is calculated as follows:

```
m = [(0.000 - 0.000) (0.7959 + 1.0969)]/2 +
    [(0.071 - 0.000) (1.0969 + 1.3979)]/2 +
    [(0.620 - 0.071) (1.3979 + 1.6990)]/2 +
    [(1.000 - 0.620) (1.6990 + 2.0000)]/2

  = 1.64147
```

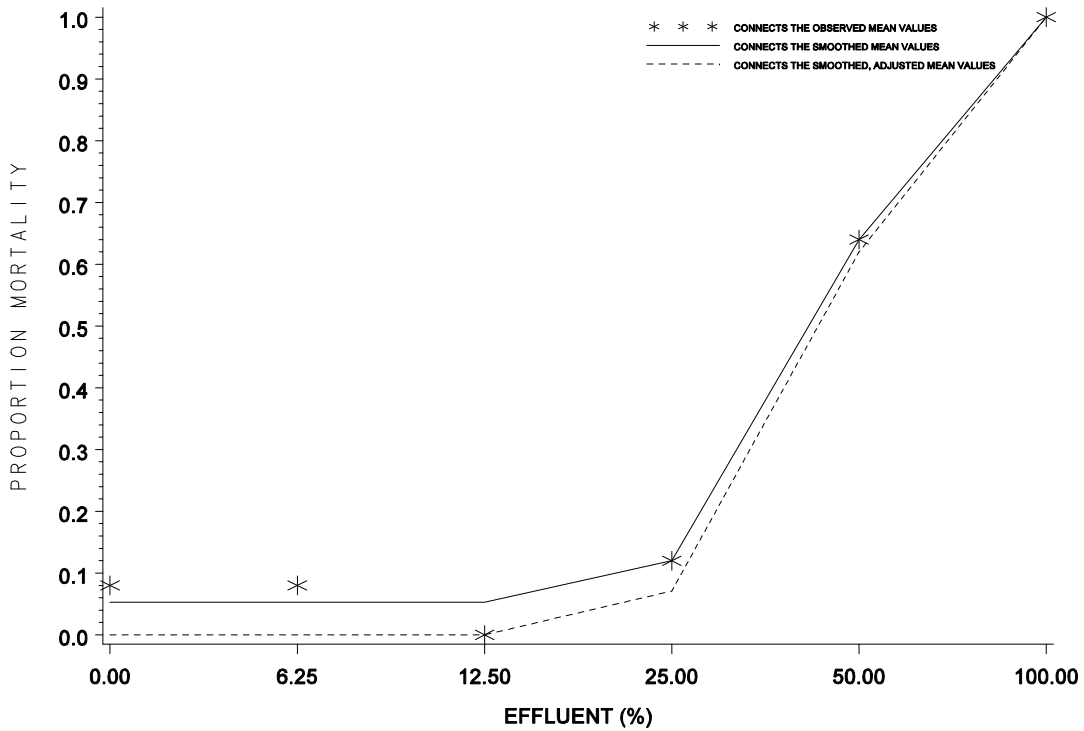

Figure I.1. Plot of observed, smoothed, and adjusted response proportions for mysid, Holmesimysis costata, survival data.

10. Calculate the estimated variance of m as follows:

$$V(m) = \sum_{i=2}^{k-1} \frac{p_i^a(1-p_i^a)(X_{i+1}-X_{i-1})^2}{4(n_i-1)}$$

Where: X_i = the $\log_{10}$ of concentration i

n_i = the number of organisms tested at effluent concentration i

p_i^a = the smoothed adjusted observed proportion mortality at effluent concentration i

k = the number of effluent concentrations tested, not including the control.

10.1 For this example, the estimated variance of m, V(m), is calculated as follows:

$$\begin{aligned} V(m) = {} & (0.000)(1.000)(1.3979 - 0.7959)^2/4(24) + \\ & (0.071)(0.929)(1.6990 - 1.0969)^2/4(24) + \\ & (0.620)(0.380)(2.0000 - 1.3979)^2/4(24) \\ = {} & 0.0011388 \end{aligned}$$

11. Calculate the 95% confidence interval for m:

$$m \pm 2.0\sqrt{V(m)}$$

11.1 For this example, the 95% confidence interval for m is calculated as follows:

$$1.64147 \pm 2\sqrt{0.0011388} = (1.57398, 1.70896)$$

12. The estimated LC50 and a 95% confidence interval for the estimated LC50 can be found by taking base_{10} antilogs of the above values.

12.1 For this example, the estimated LC50 is calculated as follows:

$$LC50 = antilog(m) = antilog(1.64147) = 43.8\%.$$

12.2 The limits of the 95% confidence interval for the estimated LC50 are calculated by taking the antilogs of the upper and lower limits of the 95% confidence interval for m as follows:

lower limit: antilog(1.57398) = 37.5%

upper limit: antilog(1.70896) = 51.2%

APPENDIX J

TRIMMED SPEARMAN-KARBER METHOD

1. The Trimmed Spearman-Karber Method is a modification of the Spearman-Karber Method, a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Hamilton, et al, 1977). The Trimmed Spearman-Karber Method estimates the trimmed mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the trimmed mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Trimmed Spearman-Karber Method is recommended only when the requirements for the Probit Analysis and the Spearman-Karber Method are not met.

4. To calculate the LC50 using the Trimmed Spearman-Karber Method, the smoothed, adjusted, observed proportion mortalities must bracket 0.5.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed, adjusted, observed proportion mortalities must be between zero and one.

6. Let p_0, p_1, ..., p_k denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy p_0 # p_1 # ... # p_k. The smoothing process replaces any adjacent p_i's that do not conform to p_0 # p_1 # ... # p_k, with their average. For example, if p_i is less than p_{i-1} then:

$$p^s_{i-1} = p^s_i = (p_i + p_{i-1})/2$$

Where: p^s_i = the smoothed observed proportion mortality for effluent concentration i.

7. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

8. Calculate the amount of trim to use in the estimation of the LC50 as follows:

$$\text{Trim} = \max(p_1^a, 1-p_k^a)$$

Where: p_1^a = the smoothed, adjusted proportion mortality for the lowest effluent concentration, exclusive of the control

p_k^a = the smoothed, adjusted proportion mortality for the highest effluent concentration

k = the number of effluent concentrations, exclusive of the control.

The minimum trim should be calculated for each data set rather than using a fixed amount of trim for each data set.

9. Due to the intensive nature of the calculation for the estimated LC50 and the calculation of the associated 95% confidence interval using the Trimmed Spearman-Karber Method, it is recommended that the data be analyzed by computer.

10. A computer program which estimates the LC50 and associated 95% confidence interval using the Trimmed Spearman-Karber Method, can be obtained through EMSL, 3411 Church Street, Cincinnati, OH 45244. The program can be obtained from EMSL-Cincinnati by sending a written request to the above address.

11. The Trimmed Spearman-Karber program automatically performs the following functions:

a. Smoothing.
b. Adjustment for mortality in the control.
c. Calculation of the necessary trim.
d. Calculation of the LC50.
e. Calculation of the associated 95% confidence interval.

12. To illustrate the Trimmed Spearman-Karber method using the Trimmed Spearman-Karber computer program, a set of data from a Topsmelt Larval Survival and Growth test will be used. The data are listed in Table J.1.

TABLE J.1. EXAMPLE OF TRIMMED SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A TOPSMELT LARVAL SURVIVAL AND GROWTH TEST (25 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	*Number of Mortalities*	*Mortality Proportion*
Control	*0*	*0.00*
6.25	*2*	*0.08*
12.5	*1*	*0.04*
25.0	*5*	*0.20*
50.0	*25*	*1.00*
100.0	*25*	*1.00*

12.1 The program requests the following input (Figure J.1):
a. Output destination (D = disk file or P = printer).
b. Control data.
c. Data for each toxicant concentration.

12.2 The program output includes the following (Figure J.2):
a. A table of the concentrations tested, number of organisms exposed, and the mortalities.
b. The amount of trim used in the calculation.
c. The estimated LC50 and the associated 95% confidence interval.

```
A:>TSK

TRIMMED SPEARMAN-KARBER METHOD.  VERSION 1.5
ENTER DATE OF TEST:
1
 ENTER TEST NUMBER:
2
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
L
ENTER TEST SPECIES NAME:
Topsmelt
ENTER TOXICANT  NAME:
effluent
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
%
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
25
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
0
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL;  MAX = 10):
5
ENTER THE  5 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):
6.25  12.5  25  50  100
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
25
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
Days
ENTER DURATION OF TEST:
7
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:
2 1 5 25 25
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
y
```

Figure J.1. Example input for Trimmed Spearman-Karber Method.

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

```
 DATE:   1                  TEST NUMBER: 2            DURATION:    7 Days
 TOXICANT:  effluent
 SPECIES:   Topsmelt

 RAW DATA:  Concentration       Number      Mortalities
 --- ----   (%)                 Exposed
                .00                 25           0
               6.25                 25           2
              12.50                 25           1
              25.00                 25           5
              50.00                 25          25
             100.00                 25          25

  SPEARMAN-KARBER TRIM:             6.00%

  SPEARMAN-KARBER ESTIMATES:     LC50:          30.98
                 95% LOWER CONFIDENCE:          27.17
                 95% UPPER CONFIDENCE:          35.32

 NOTE:  MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
        ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.
-------------------------------------------------------------------------------
```

Figure J.2. Example output for Trimmed Spearman-Karber Method.

APPENDIX K

GRAPHICAL METHOD

1. The Graphical Method is used to calculate the LC50. It is a mathematical procedure which estimates the LC50 by linearly interpolating between points of a plot of observed percent mortality versus the base 10 logarithm ($\log_{10}$) of percent effluent concentration. This method does not provide a confidence interval for the LC50 estimate and its use is only recommended when there are no partial mortalities after the data is smoothed and adjusted for control mortality. The only requirement for the Graphical Method is that the observed percent mortalities bracket 50%.

2. For an analysis using the Graphical Method the data must first be smoothed and adjusted for mortality in the control replicates. The procedure for smoothing and adjusting the data is detailed in the following steps.

3. The Graphical Method is illustrated below using a set of mortality data from a Topsmelt Larval Survival and Growth test. These data are listed in Table K.1.

TABLE K.1. EXAMPLE OF GRAPHICAL METHOD: MORTALITY DATA FROM A TOPSMELT LARVAL SURVIVAL AND GROWTH TEST (25 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	*Number of Mortalities*	*Mortality Proportion*
Control	*1*	*0.04*
6.25	*0*	*0.00*
12.5	*0*	*0.00*
25.0	*0*	*0.00*
50.0	*25*	*1.00*
100.0	*25*	*1.00*

4. Let p_0, p_1, ..., p_k denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy p_0 # p_1 # ... # p_k. The smoothing process replaces any adjacent p_i's that do not conform to p_0 # p_1 # ... # p_k with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i\&1}^{s} \text{ ' } p_i^{s} \text{ ' } (p_i\%p_{i\&1})/2$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

4.1 For the data in this example, because the observed mortality proportions for the 6.25%, 12.5%, and 25.0% effluent concentrations are less than the observed response proportion for the control, the values for these four groups must be averaged:

$$p_0^s \text{ ' } p_1^s \text{ ' } p_2^s \text{ ' } p_3^s \text{ ' } \frac{0.04 \% 0.00 \% 0.00 \% 0.00}{4} \text{ ' } \frac{0.04}{4} \text{ ' } 0.01$$

4.2 Since p_4 = p_5 = 1.00 are larger then 0.01, set p_4^s = p_5^s = 1.00. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table K.2.

5. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a \text{ ' } (p_i^s\&p_0^s) /(1\&p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

5.1 Because the smoothed observed proportion mortality for the control group is greater than zero, the responses must be adjusted using Abbott's formula, as follows:

$$p_0^a = p_1^a = p_2^a = p_3^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.01 - 0.01}{1 - 0.0125} = \frac{0.0}{0.99} = 0.0$$

$$p_4^a = p_5^a = \frac{p_4^s - p_0^s}{1 - p_0^s} = \frac{1.00 - 0.01}{1 - 0.01} = \frac{0.99}{0.99} = 1.00$$

A table of the smoothed, adjusted response proportions for the effluent concentrations is shown in Table K.2.

TABLE K.2. EXAMPLE OF GRAPHICAL METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A TOPSMELT LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration %	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.04	0.01	0.00
6.25	0.00	0.01	0.00
12.5	0.00	0.01	0.00
25.0	0.00	0.01	0.00
50.0	1.00	1.00	1.00
100.0	1.00	1.00	1.00

5.2 Plot the smoothed, adjusted data on 2-cycle semi-log graph paper with the logarithmic axis (the y axis) used for percent effluent concentration and the linear axis (the x axis) used for observed percent mortality. A plot of the smoothed, adjusted data is shown in Figure K.1.

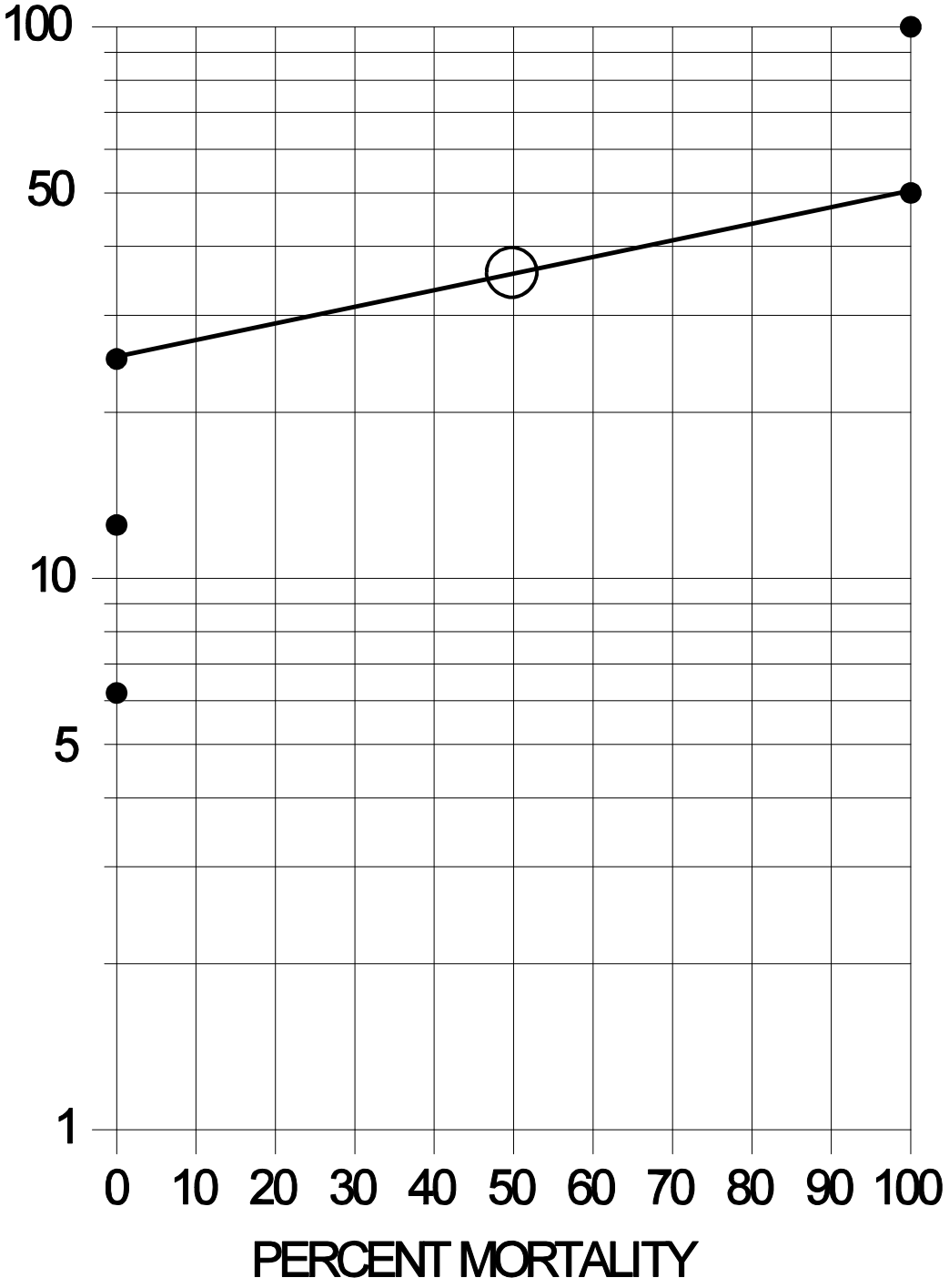

Figure K.1. Plot of the smoothed adjusted response proportions for topsmelt, Atherinops affinis, survival data.

6. Locate the two points on the graph which bracket 50% mortality and connect them with a straight line.

7. On the scale for percent effluent concentration, read the value for the point where the plotted line and the 50% mortality line intersect. This value is the estimated LC50 expressed as a percent effluent concentration.

7.1 For this example, the two points on the graph which bracket the 50% mortality line (0% mortality at 25% effluent, and 100% mortality at 50% effluent) are connected with a straight line. The point at which the plotted line intersects the 50% mortality line is the estimated LC50. The estimated LC50 = 35% effluent.

APPENDIX L

LINEAR INTERPOLATION METHOD

1. GENERAL PROCEDURE

1.1 The Linear Interpolation Method is used to calculate a point estimate of the effluent or other toxicant concentration that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms (Inhibition Concentration, or IC). The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests, and the generation of an endpoint from a continuous model that allows a traditional quantitative assessment of the precision of the endpoint, such as confidence limits for the endpoint of a single test, and a mean and coefficient of variation for the endpoints of multiple tests.

1.2 The Linear Interpolation Method assumes that the responses (1) are monotonically non-increasing, where the mean response for each higher concentration is less than or equal to the mean response for the previous concentration, (2) follow a piecewise linear response function, and (3) are from a random, independent, and representative sample of test data. If the data are not monotonically non-increasing, they are adjusted by smoothing (averaging). In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean. Also, no assumption is made about the distribution of the data except that the data within a group being resampled are independent and identically distributed.

2. DATA SUMMARY AND PLOTS

2.1 Calculate the mean responses for the control and each toxicant concentration, construct a summary table, and plot the data.

3. MONOTONICITY

3.1 If the assumption of monotonicity of test results is met, the observed response means ($\bar{x}_i$) should stay the same or decrease

as the toxicant concentration increases. If the means do not decrease monotonically, the responses are "smoothed" by averaging (pooling) adjacent means.

3.2 Observed means at each concentration are considered in order of increasing concentration, starting with the control mean (**&**$_1$). If the mean observed response at the lowest toxicant concentration (**&**$_2$) is equal to or smaller than the control mean (**&**$_1$), it is used as the response. If it is larger than the control mean, it is averaged with the control, and this average is used for both the control response (M_1) and the lowest toxicant concentration response (M_2). This mean is then compared to the mean observed response for the next higher toxicant concentration (**&**$_3$). Again, if the mean observed response for the next higher toxicant concentration is smaller than the mean of the control and the lowest toxicant concentration, it is used as the response. If it is higher than the mean of the first two, it is averaged with the first two, and the mean is used as the response for the control and two lowest concentrations of toxicant. This process is continued for data from the remaining toxicant concentrations. A numerical example of smoothing the data is provided below. (Note: Unusual patterns in the deviations from monotonicity may require an additional step of smoothing). Where **&**$_i$ decrease monotonically, the **&**$_i$ become M_i without smoothing.

4. **LINEAR INTERPOLATION METHOD**

4.1 The method assumes a linear response from one concentration to the next. Thus, the ICp is estimated by linear interpolation between two concentrations whose responses bracket the response of interest, the (p) percent reduction from the control.

4.2 To obtain the estimate, determine the concentrations C_J and C_{J+1} which bracket the response M_1 (1 - p/100), where M_1 is the smoothed control mean response and p is the percent reduction in response relative to the control response. These calculations can easily be done by hand or with a computer program as described below. The linear interpolation estimate is calculated as follows:

$$ICp = C_J + [M_1 (1 - p/100) - M_J] \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

Where:	C_J	=	tested concentration whose observed mean response is greater than $M_1(1 - p/100)$.
	C_{J+1}	=	tested concentration whose observed mean response is less than $M_1(1 - p/100)$.
	M_1	=	smoothed mean response for the control.
	M_J	=	smoothed mean response for concentration J.
	M_{J+1}	=	smoothed mean response for concentration J + 1.
	p	=	percent reduction in response relative to the control response.
	IC*p*	=	estimated concentration at which there is a percent reduction from the smoothed mean control response. The IC*p* is reported for the test, together with the 95% confidence interval calculated by the ICPIN.EXE program described below.

4.3 If the C_J is the highest concentration tested, the IC*p* would be specified as *greater than* C_J. If the response at the lowest concentration tested is used to extrapolate the ICp value, the ICp should be expressed as a *less than the lowest test concentration.*

5. **CONFIDENCE INTERVALS**

5.1 Due to the use of a linear interpolation technique to calculate an estimate of the ICp, standard statistical methods for calculating confidence intervals are not applicable for the ICp. This limitation is avoided by use a technique known as the bootstrap method as proposed by Efron (1982) for deriving point estimates and confidence intervals.

5.2 In the Linear Interpolation Method, the smoothed response means are used to obtain the ICp estimate reported for the test. The bootstrap method is used to obtain the 95% confidence interval for the true mean. In the bootstrap method, the test data Y_{ji} is randomly resampled with replacement to produce a new set of data Y_{ji}*, that is statistically equivalent to the original data, but a new and slightly different estimate of the IC*p* (IC*p**) is obtained. This process is repeated at least 80 times (Marcus and Holtzman, 1988) resulting in multiple "data" sets, each with an associate ICp* estimate. The distribution of the ICp* estimates derived from the sets of resampled data approximates the sampling distribution of the ICp estimate. The standard error of the ICp is estimated by the standard deviation of the individual ICp* estimates. Empirical confidence intervals are derived from the quantiles of the ICp* empirical distribution. For example, if the test data are resampled a minimum of 80 times, the empirical 2.5% and the 97.5% confidence limits are approximately the second smallest and second largest ICp* estimates (Marcus and Holtzman, 1988).

5.3 The width of the confidence intervals calculated by the bootstrap method is related to the variability of the data. When confidence intervals are wide, the reliability of the IC estimate is in question. However, narrow intervals do not necessarily indicate that the estimate is highly reliable, because of undetected violations of assumptions and the fact that the confidence limits based on the empirical quantiles of a bootstrap distribution of 80 samples may be unstable.

5.4 The bootstrapping method of calculating confidence intervals is computationally intensive. For this reason, all of the calculations associated with determining the confidence intervals for the ICp estimate have been incorporated into a computer program. Computations are most easily done with a computer program such as the revision of the BOOTSTRP program (USEPA, 1988; USEPA, 1989) which is now called "ICPIN" and is described below in subsection 7.

6. **MANUAL CALCULATIONS**

6.1 DATA SUMMARY AND PLOTS

6.1.1 The data used in this example are the mysid growth data used in the example in Section 14. The data is presented as the mean weight per surviving organism. Table L.1 includes the raw data and the mean growth for each concentration. A plot of the data is provided in Figure L.1.

6.2 MONOTONICITY

6.2.1. As seen in the table, the observed means are monotonically non-increasing with respect to concentration. Therefore, the smoothed means will be simply the corresponding observed mean. The observed means are represented by $\&_i$ and the smoothed means by M_i. Table L.2 contains the smoothed means and Figure L.1 gives a plot of the smoothed response curve.

6.3 LINEAR INTERPOLATION

6.3.1 An estimates of the IC25 can be calculated using the Linear Interpolation Method. A 25% reduction in mean weight, compared to the controls, would result in a mean weight of 0.039, where $M_1(1-p/100) = 0.052(1-25/100)$. Examining the smoothed means and their associated concentrations (Table L.2), the response, 0.039 mg, is bracketed by C_4 = 5.60% and and C_5 = 10.0%.

TABLE L.1. MYSID, HOLMESIMYSIS COSTATA, GROWTH DATA

		Concentration (%)			
Replicate	*Control*	*1.80*	*3.20*	*5.60*	*10.0*
1	*0.048*	*0.055*	*0.057*	*0.041*	*0.033*
2	*0.058*	*0.048*	*0.050*	*0.040*	*0.000*
3	*0.047*	*0.042*	*0.046*	*0.041*	*0.000*
4	*0.058*	*0.041*	*0.043*	*0.043*	*0.000*
5	*0.051*	*0.052*	*0.045*	*0.040*	*0.000*
Mean($\&_i$)	*0.052*	*0.048*	*0.048*	*0.041*	*0.007*
i	*1*	*2*	*3*	*4*	*5*

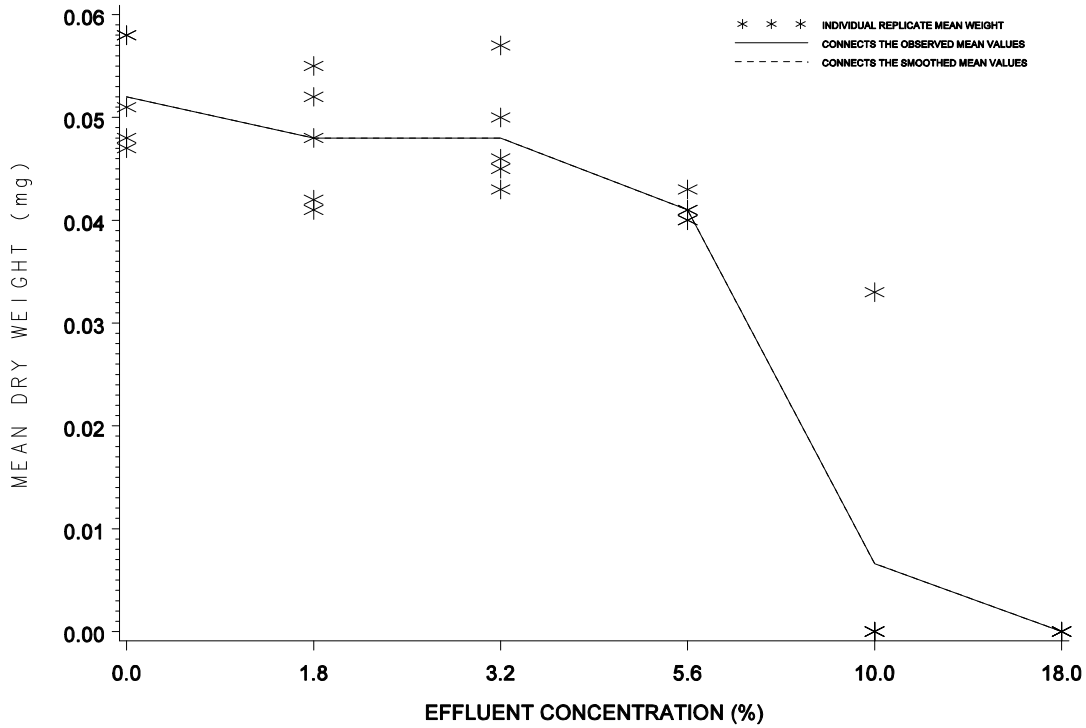

Figure L.1. Plot of raw data, observed means, and smoothed means for the mysid, Holmesimsys costata, growth data.

TABLE L.2. MYSID, HOLMESIMYSIS COSTATA, MEAN GROWTH RESPONSE AFTER SMOOTHING

Toxicant Conc. (%)	i	Response Means Y_i (mg)	Smoothed Means M_i (mg)
Control	1	0.052	0.052
1.80	2	0.048	0.048
3.20	3	0.048	0.048
5.60	4	0.041	0.041
10.00	5	0.007	0.007
18.00	6	0.000	0.000

6.3.2 Using the equation from section 4.2, the estimate of the IC25 is calculated as follows:

$$ICp = C_J + [M_1 (1 - p/100) - M_J] \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

$$IC25 = 5.60 + [0.052 (1 - 25/100) - 0.041] \frac{(10.0 - 5.60)}{(0.007 - 0.041)}$$

$$= 5.86\%$$

6.4 CONFIDENCE INTERVALS

6.4.1 Confidence intervals for the ICp are derived using the bootstrap method. As described above, this method involves randomly resampling the individual observations and recalculating the ICp at least 80 times, and determining the mean ICp, standard deviation, and empirical 95% confidence intervals. For this reason, the confidence intervals are calculated using a computer program called ICPIN. This program is described below and is available to carry out all the calculations of both the interpolation estimate (ICp) and the confidence intervals.

7. **COMPUTER CALCULATIONS**

7.1 The computer program, ICPIN, prepared for the Linear Interpolation Methods was written in TURBO PASCAL for IBM compatible PCs. The program (version 2.0) has been modified by Computer Science Corporation, Duluth, MN with funding provided by the Environmental Research Laboratory, Duluth, MN (Norberg-King, 1993). The program was originally developed by Battelle Laboratories, Columbus, OH through a government contract supported by the Environmental Research Laboratory, Duluth, MN (USEPA, 1988). A compiled, executable version of the program and supporting documentation can be obtained by sending a written request to EMSL-Cincinnati, 3411 Church Street, Cincinnati, OH 45244.

7.2 The ICPIN.EXE program performs the following functions: 1) it calculates the observed response means ($\overline{Y}_i$) (response means); 2) it calculates the standard deviations; 3) checks the responses for monotonicity; 4) calculates smoothed means (M_i) (pooled response means) if necessary; 5) uses the means, M_i, to calculate the initial ICp of choice by linear interpolation; 6) performs a user-specified number of bootstrap resamples between 80 and 1000 (as multiples of 40); 7) calculates the mean and standard deviation of the bootstrapped ICp estimates; and 8) provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven (Norberg-King, 1993).

7.3 For the IC*p* calculation, up to twelve treatments can be input (which includes the control). There can be up to 40 replicates per concentration, and the program does not require an equal number of replicates per concentration. The value of p can range from 1% to 99%.

7.4 DATA INPUT

7.4.1 Data is entered directly into the program onscreen. A sample data entry screen in shown in Figure L.2. The program documentation provides guidance on the entering and analysis of data for the Linear Interpolation Method.

```
ICp Data Entry/Edit Screen                          Current File:
Conc. ID        *   1       *     2     *     3     *     4     *   5       *  6
Conc. Tested    *           *           *           *           *           *
Response  1     *           *           *           *           *           *
Response  2     *           *           *           *           *           *
Response  3     *           *           *           *           *           *
Response  4     *           *           *           *           *           *
Response  5     *           *           *           *           *           *
Response  6     *           *           *           *           *           *
Response  7     *           *           *           *           *           *
Response  8     *           *           *           *           *           *
Response  9     *           *           *           *           *           *
Response 10     *           *           *           *           *           *
Response 11     *           *           *           *           *           *
Response 12     *           *           *           *           *           *
Response 13     *           *           *           *           *           *
Response 14     *           *           *           *           *           *
Response 15     *           *           *           *           *           *
Response 16     *           *           *           *           *           *
Response 17     *           *           *           *           *           *
Response 18     *           *           *           *           *           *
Response 19     *           *           *           *           *           *
Response 20     *           *           *           *           *           *

4444444444444444444444444444444444444444444444444444444444444444444444444444
F10 for Command Menu                    Use Arrow Keys to Switch Fields
```

Figure L.2. ICp data entry/edit screen. Twelve concentration identifications can be used. Data for concentrations are entered in columns 1 through 6. For concentrations

7.4.2 The user selects the ICp estimate desired (e.g., IC25 or IC50) and the number of resamples to be taken for the bootstrap method of calculating the confidence intervals. The program has the capability of performing any number of resamples from 80 to 1000 as multiples of 40. However, Marcus and Holtzman (1988) recommend a minimum of 80 resamples for the bootstrap method be used and at least 250 resamples are better (Norberg-King, 1993).

7.5 DATA OUTPUT

7.5.1 The program output includes the following (see Figure L.3)

1. A table of the concentration identification, the concentration tested and raw data response for each replicate and concentration.
2. A table of test concentrations, number of replicates, concentration (units), response means (Y_i), standard deviations for each response mean, and the pooled response means (smoothed means; M_i).
3. The linear interpolation estimate of the ICp using the means (M_i). *Use this value for the ICp estimate.*
4. The mean ICp and standard deviation from the bootstrap resampling.
5. The confidence intervals calculated by the bootstrap method for the ICp. Provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven.

7.6 ICPIN program output for the analysis of the mysid growth data in Table L.1 is provided in Figure L.3.

7.6.1 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 5.8174%. The empirical 95% confidence intervals for the true mean was 4.9440% to 6.2553%.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.80	3.20	5.60	10.0	18.0
Response 1	.048	.055	.057	.041	.033	0
Response 2	.058	.048	.050	.040	0	0
Response 3	.047	.042	.046	.041	0	0
Response 4	.058	.041	.043	.043	0	0
Response 5	.051	.052	.045	.040	0	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: mysid, Holmesimysis costata
Test Duration: 7 days
DATA FILE: mysid.icp
OUTPUT FILE: mysid.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	5	0.000	0.052	0.005	0.052
2	5	1.800	0.048	0.006	0.048
3	5	3.200	0.048	0.006	0.048
4	5	5.600	0.041	0.001	0.041
5	5	10.000	0.007	0.015	0.007
6	5	18.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 5.8174 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 5.8205 Standard Deviation: 0.2673
Original Confidence Limits: Lower: 4.9440 Upper: 6.2553
Expanded Confidence Limits: Lower: 4.5073 Upper: 6.4743
Resampling time in Seconds: 0.22 Random_Seed: 526805435

Figure L.3. Example of ICPIN program output for the IC25.

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