SECTION 11

ACUTE TOXICITY DATA ANALYSIS

11.1 INTRODUCTION

11.1.1 The objective of acute toxicity tests with effluents and receiving waters is to identify discharges of toxic effluents in acutely toxic amounts. Data are derived from tests designed to determine the adverse effects of effluents and receiving waters on the survival of the test organisms. The recommended effluent toxicity test consists of a control and five or more concentrations of effluent (i.e., multi-effluent-concentration, or definitive tests), in which the endpoint is (1) an estimate of the effluent concentration which is lethal to 50% of the test organisms in the time period prescribed by the test, expressed as the LC50, or (2) the highest effluent concentration at which survival is not significantly different from the control (No-Observed-Adverse-Effect Concentration, or NOAEC). Receiving water tests may be single concentration or multi-concentration tests. The LC50 is determined by the Graphical, Spearman-Karber, Trimmed Spearman-Karber, or Probit Method. The NOAEC is determined by hypothesis testing.

11.1.2 Some states require tests consisting of a control and a single concentration of effluent with a pass/fail endpoint. Control survival must be 90% or greater for an acceptable test. The test "passes" if survival in the control and effluent concentration equals or exceeds 90%. The test "fails" if survival in the effluent is less than 90%, and is significantly different from control survival (which must be 90% or greater), as determined by hypothesis testing.

11.1.3 The toxicity of receiving (surface) water can be determined with (1) a paired test consisting of four replicates each of a suitable control and 100% surface water, or (2) a multi-concentration test. The results of the first type of test (100% receiving water and a control) are analyzed by hypothesis testing. The results of the second type of test may be analyzed by hypothesis testing or used to determine an LC50.

11.1.4 The data analysis methods recommended in this manual have been chosen primarily because they are (1) well-tested and well-documented, (2) applicable to most types of test data sets for which they are recommended, but still powerful, and (3) most easily understood by non-statisticians. Many other methods were considered in the selection process, and it is recognized that the methods selected are not the only possible methods of analysis of acute toxicity data.

11.1.5 ROLE OF THE STATISTICIAN

11.1.5.1 The use of the statistical methods described in this manual for routine data analysis does not require the assistance of a statistician. However, if the data appear unusual in any way, or fail to meet the necessary assumptions, a statistician should be consulted. The choice of a statistical method to analyze toxicity test data and the interpretation of the results of the analysis of the data can become problematic if there are anomalies in the data. Analysts who are not proficient in statistics are strongly advised to seek the assistance of a statistician before selecting alternative methods of analysis and using the results.

11.1.6 INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

11.1.6.1 A critical assumption in the statistical analysis of toxicity data is statistical independence among observations. 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. One of the best ways to insure independence is to properly follow randomization procedures. The purpose of randomization is to avoid situations where test organisms are placed serially, by level of concentration, 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.

11.1.6.2 Another area for potential bias of results is the presence of outliers. 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 statistical analysis should be performed both with and without the outlier, and the results of both analyses should be reported. For a discussion of techniques for evaluating outliers, see Draper and John (1981).

11.2 DETERMINATION OF THE LC50 FROM DEFINITIVE, MULTI-EFFLUENT-CONCENTRATION ACUTE TOXICITY TESTS

11.2.1 The method used to estimate the LC50 from multi-concentration acute toxicity tests depends on the shape of the tolerance distribution, and how well the effluent concentrations chosen characterize the cumulative distribution function for the tolerance distribution (i.e., the number of partial mortalities). A review of effluent acute toxicity data from the last 248 tests performed by the Ecological Support Branch, Environmental Services Division, EPA Region 4, indicated the following pattern in the number of partial mortalities: (1) no partial mortalities (all or nothing response) - 28%; (2) one partial mortality - 54%; (3) two or more partial mortalities - 16%; (4) LC50 occurring a one of the test concentrations - 2%.

11.2.1.1 Four methods for estimating the LC50 are presented below: the Graphical Method, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, and the Probit Method. The analysis scheme is shown in Figure 6. Included in the presentation of each method is a description of the method, the requirements for the method, a description of the calculations involved in the method or a description of the computer program, and an example of the calculations.

11.2.1.2 The Probit Method, the Spearman-Karber Method, and the Trimmed Spearman-Karber Method are designed to produce LC50 values and associated 95% confidence intervals. It should be noted that software used to calculate point estimates occasionally may not provide associated 95% confidence intervals. This situation may arise when test data do not meet specific assumptions required by the statistical methods, when point estimates are outside of the test concentration range, and when specific limitations imposed by the software are encountered. USEPA (2000a) provides guidance on confidence intervals under these circumstances.

11.2.2 THE GRAPHICAL METHOD

11.2.2.1 Description

1. The Graphical Method is a mathematical procedure for calculating the LC50.

2. The procedure 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.

3. It does not provide a confidence interval for the LC50 estimate.

4. Use of the Graphical Method is only recommended when there are no partial mortalities.

11.2.2.2 Requirements

1. The only requirement for the Graphical Method is that the observed percent mortalities bracket the 50%.

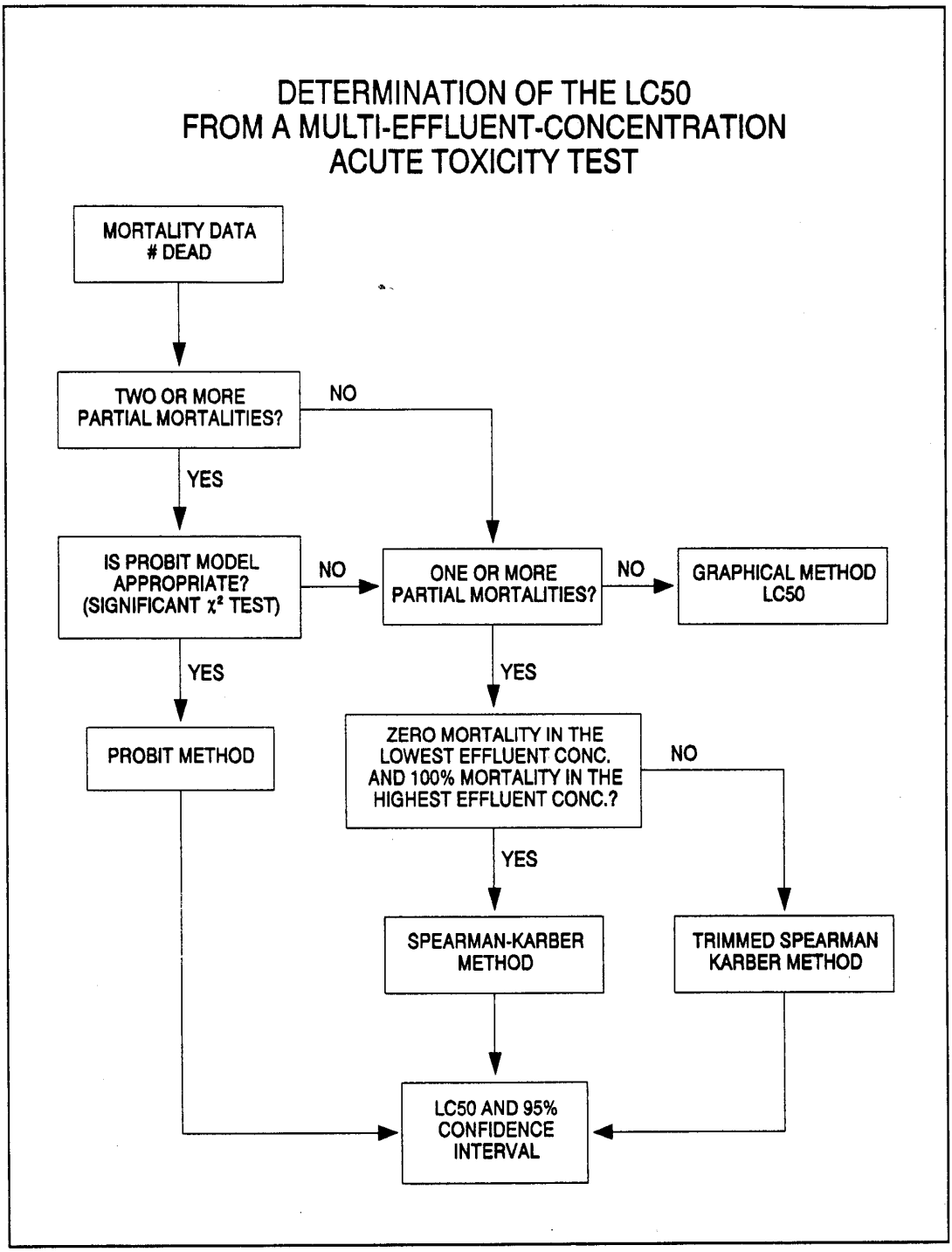

Figure 6. Flowchart for determination of the LC50 for multi-effluent-concentration acute toxicity tests.

11.2.2.3 General Procedure

1. Let $p_o, p_1, ..., p_k$ 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 $p_o \leq ... \leq p_k$. The smoothing replaces any adjacent p_i's that do not conform to $p_o \leq ... \leq p_k$, with their average. For example, if p_i is less than p_{i-1}, then:

$$p_{i-1}^s = p_i^s = (p_i + p_{i-1})/2$$

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

2. 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_o^s = the smoothed observed proportion mortality for the control.

3. 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.

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

5. 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.

11.2.2.4 Example Calculation

1. All-or-nothing data (Graphical Method) in Table 20 are used in the calculations. Note that in this case, the data must be smoothed and adjusted for mortality in the controls.

2. To smooth the data, the observed proportion mortality for the control and the lower three effluent concentrations must be averaged. The smoothed observed proportion mortalities are as follows: 0.0125, 0.0125, 0.0125, 0.0125, 1.0, and 1.0.

3. The smoothed responses are adjusted for control mortality (see 11.2.2.3), where the smoothed response for the control (p_o^s) = 0.0125. The smoothed, adjusted response proportions for the effluent concentrations are as follows: 0.0, 0.0, 0.0, 1.0, and 1.0.

4. A plot of the smoothed, adjusted data is shown in Figure 7.

5. 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.

6. The point at which the plotted line intersects the 50% mortality line is the estimated LC50. The estimated LC50 = 35% effluent.

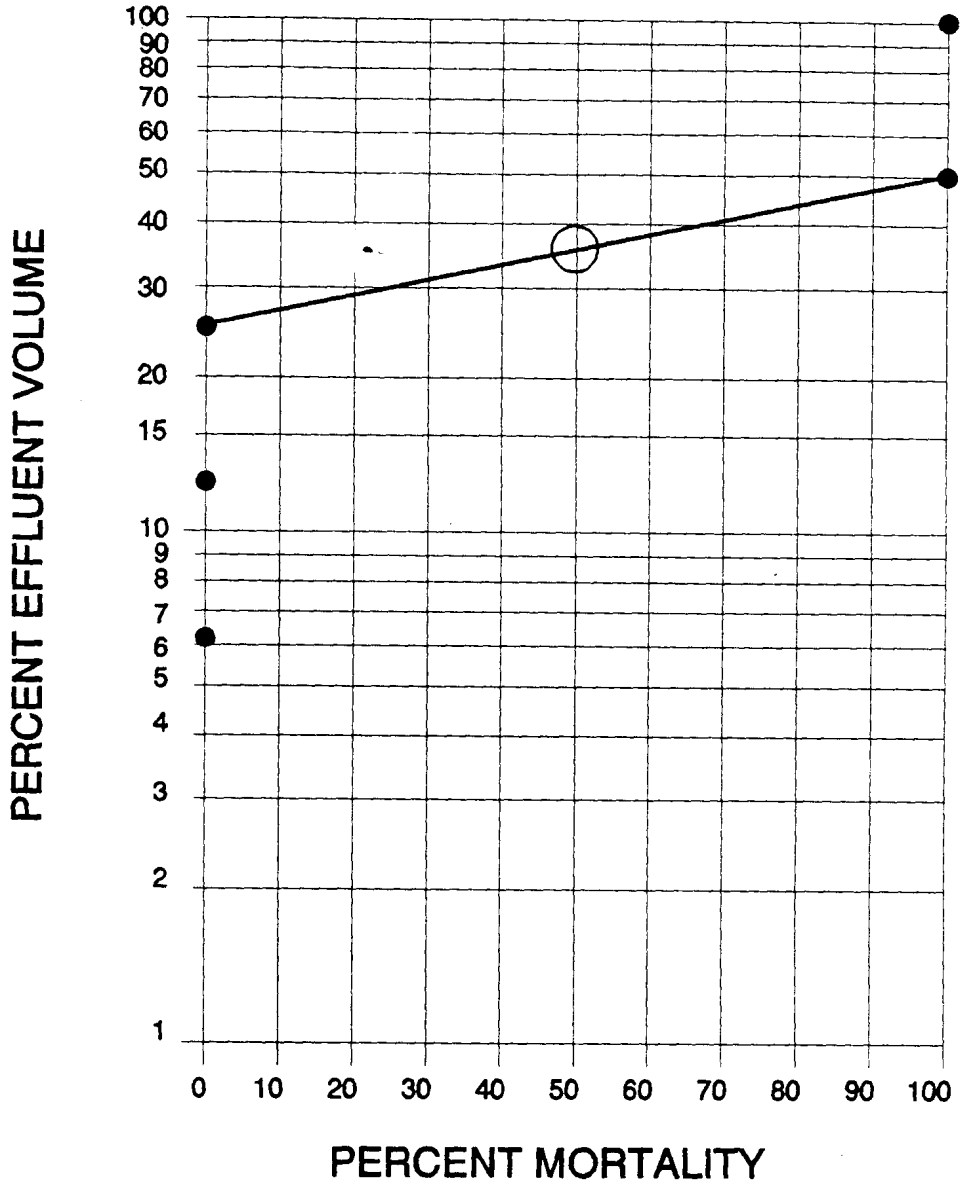

Figure 7. Plotted data and fitted line for graphical method, using all-or-nothing data.

TABLE 20. MORTALITY DATA (NUMBER OF DEAD ORGANISMS) FROM ACUTE TOXICITY TESTS USED IN EXAMPLES OF LC50 DETERMINATIONS (20 ORGANISMS IN THE CONTROL AND ALL TEST CONCENTRATIONS)

Effluent Conc. (%)	Method of Analysis			
	Graphical	Spearman-Karber	Trimmed Spearman-Karber	Probit
CONTROL	1	1	1	0
6.25%	0	1	0	0
12.5%	0	0	2	3
25.0%	0	0	0	9
50.0%	20	13	0	20
100.0%	20	20	16	20

11.2.3 THE SPEARMAN-KARBER METHOD

11.2.3.1 Description

1. The Spearman-Karber Method is a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978).

2. This procedure 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.

3. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data are smoothed.

4. Abbott's procedure is used to "adjust" the test results for mortality occurring in the control.

5. 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.

11.2.3.2 Requirements

1. To calculate the LC50 estimate, the following must be true:
   a. The smoothed adjusted proportion mortality for the lowest effluent concentration (not including the control) must be zero.
   b. The smoothed adjusted proportion mortality for the highest effluent concentration must be one.

2. 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.

11.2.3.3 General Procedure

1. The first step in the estimation of the LC50 by the Spearman-Karber Method is to smooth the observed response proportions, $p_{i,}$ if they do not satisfy $p_{o} \leq \ldots \leq p_{k}$ (see 11.2.2.3, Step 1).

2. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (see 11.2.2.3, Step 2).

3. 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.

4. 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.

5. 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.

6. Calculate the 95% confidence interval for m: $m \pm 2.0 \sqrt{V(m)}$

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

8. With the exclusion of the plot in item 3, the above calculations can be carried out using the Trimmed Spearman-Karber computer program mentioned in 11.2.4.3 and 11.2.4.4.

11.2.3.4 Example Calculation

1. Mortality data from a definitive, multi-concentration, acute toxicity test are given in Table 20. Note that the data must be smoothed and adjusted for mortality in the controls.

2. To smooth the data, the observed proportion mortality for the control, and the observed proportion mortality for the 6.25%, 12.5%, and 25% effluent concentrations must be averaged. The smoothed observed proportion mortalities are as follows: 0.025, 0.025, 0.025, 0.025, 0.65, and 1.00.

3. To adjust the smoothed, observed proportion mortality in each effluent concentration for mortality in the control group, Abbott's formula must be used. After smoothing and adjusting, the proportion mortalities for the effluent concentrations are as follows: 0.000, 0.000, 0.000; 0.641, and 1.000.

4. The data will not be plotted for this example. For an example of the plotting procedures, see 11.2.2.4.

5. The $\log_{10}$ of the estimated LC50, m, is calculated as follows:

$$\begin{aligned} m &= [(0.0000 - 0.0000)(0.7959 + 1.0969)]/2 + \\ &\quad [(0.0000 - 0.0000)(1.0969 + 1.3979)]/2 + \\ &\quad [(0.6410 - 0.0000)(1.3979 + 1.6990)]/2 + \\ &\quad [(1.0000 - 0.6410)(1.6990 + 2.0000)]/2 \\ &= 1.656527 \end{aligned}$$

6. The estimated variance of m, V(m), is calculated as follows:

$$\begin{aligned} V(m) &= (0.0000)(1.0000)(1.3979 - 0.7959)^2/4(19) + \\ &\quad (0.0000)(1.0000)(1.6990 - 1.0969)^2/4(19) + \\ &\quad (0.6410)(0.3590)(2.0000 - 1.3979)^2/4(19) \\ &= 0.0010977 \end{aligned}$$

7. The 95% confidence interval for m is calculated as follows:

$$1.656527 \pm 2\sqrt{0.0010977} = (1.5902639,\ 1.7227901)$$

8. The estimated LC50 is as follows: antilog(1.656527) = 45.3%.

9. The upper limit of the 95% confidence interval for the estimated LC50 is as follows:

antilog(1.7227901) = 52.8%

10. The lower limit of the 95% confidence interval for the estimated LC50 is as follows:

antilog(1.5902639) = 38.9%

11.2.4 THE TRIMMED SPEARMAN-KARBER METHOD

11.2.4.1 Description

1. The Trimmed Spearman-Karber Method is a modification of the Spearman-Karber nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Hamilton, et al, 1977).

2. This procedure 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.

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

11.2.4.2 Requirements

1. To calculate the LC50 estimate with the Trimmed Spearman-Karber Method, the smoothed, adjusted, observed proportion mortalities must bracket 0.5.

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

11.2.4.3 General Procedure

1. Smooth the observed proportion mortalities as described in 11.2.2.3, Step 1.

2. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (see 11.2.2.3, Step 2).

3. Plot the smoothed, adjusted data as described in 11.2.2.3, Step 3.

4. 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.

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

6. A computer program which estimates the LC50 and associated 95% confidence interval using the Trimmed-Karber Method, can be obtained through the Environmental Monitoring and Support Laboratory (EMSL), 26 W. Martin Luther King Drive, Cincinnati, OH 45268. The program can be obtained from EMSL-Cincinnati by sending a diskette with a written request to the above address.

7. The modified program automatically performs the following functions:
   a. Smoothing.
   b. Adjustment for mortality in the control.
   c. Calculation of the trim.
   d. Calculation of the LC50.
   e. Calculation of the associated 95% confidence interval.

11.2.4.4 Example Calculation Using the Computer Program

1. Data from Table 20 are used to illustrate the analysis using the Trimmed Spearman-Karber program.

2. The program requests the following input (see Figure 8):
   a. Output destination (D = disk file or P = printer).
   b. Title for output.
   c. Control data.
   d. Data for each toxicant concentration.

```
TRIMMED SPEARMAN-KARBER METHOD.  VERSION 1.5


ENTER DATE OF TEST:
08/19/93
ENTER TEST NUMBER:
1
WHAT IS TO BE ESTIMATED?
 (ENTER "L" FOR LC50 AND "E" FOR EC50)
L

ENTER TEST SPECIES NAME:
Fathead minnow

ENTER TOXICANT NAME:
Effluent

ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT:
%

ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
20

ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
1

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:
20

ENTER UNITS FOR DURATION OF EXPERIMENT
 (ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
H

ENTER DURATION OF TEST:
96

ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:
0 2 0 0 16

WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
Y
```

Figure 8. Example of input for computer program for Trimmed Spearman-Karber Method.

3. The program output includes the following (see Figure 9):
   a. A table of the concentrations tested, number of organisms exposed, and mortalities.
   b. The amount of trim used in the calculation.
   c. The estimated LC50 and the associated 95% confidence interval.

4. The analysis results for this example are as follows:
   a. The observed proportion mortalities smoothed and adjusted for mortality in the control.
   b. The amount of trim used to calculate the estimate:

$$\text{trim} = \max\{0.00, 0.205\} = 0.205.$$

   c. The estimate of the LC50 is 77.1% with a 95% confidence interval of (69.7%, 85.3%).

11.2.5 THE PROBIT METHOD

11.2.5.1 Description

1. The Probit Method is a parametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978).

2. The analysis consists of transforming the observed proportion mortalities with a probit transformation, and transforming the effluent concentrations to $\log_{10}$.

3. Given the assumption of normality for the $\log_{10}$ of the tolerances, the relationship between the transformed variables mentioned above is approximately linear.

4. This relationship allows estimation of linear regression parameters, using an iterative approach.

5. The estimated LC50 and associated confidence interval are calculated from the estimated linear regression parameters.

11.2.5.2 Requirements

1. To obtain a reasonably precise estimate of the LC50 with the Probit Method, the observed proportion mortalities must bracket 0.5.

2. The $\log_{10}$ of the tolerance is assumed to be normally distributed.

3. To calculate the LC50 estimate and associated 95% confidence interval, two or more of the observed proportion mortalities must be between zero and one.

11.2.5.3 General Procedure

1. Due to the intensive nature of the calculations for the estimated LC50 and associated 95% confidence interval using the Probit Method, it is recommended that the data be analyzed by a computer program.

2. A machine-readable, compiled, version of a computer program to estimate the LC1 and LC50 and associated 95% confidence intervals using the Probit Method can be obtained from EMSL-Cincinnati by sending a diskette with a written request to the Environmental Monitoring Systems Laboratory, 26 W. Martin Luther King Drive, Cincinnati, OH 45268.

```
TRIMMED SPEARMAN-KARBER METHOD, VERSION 1.5

DATE:      08/18/93          TEST NUMBER: 1          DURATION:   96 H
TOXICANT:  Effluent
SPECIES:   Fathead minnow


RAW DATA:  Concentration      Number          Mortalities
--- ----   (%)               Exposed


             .00               20               1
            6.25               20               0
           12.50               20               2
           25.00               20               0
           50.00               20               0
          100.00               20              16


SPEARMAN-KARBER TRIM:   20.51%

SPEARMAN-KARBER ESTIMATES:                  LC50:  77.11
                          95% Lower Confidence:  69.74
                          95% Upper Confidence:  85.26

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

WOULD YOU LIKE TO HAVE A COPY SENT TO THE PRINTER(Y/N)?
```

Figure 9. Example of output from computer program for Trimmed Spearman-Karber Method.

11.2.5.4 Example Using the Computer Program

1. Data from Table 20 are used to illustrate the operation of the Probit program for calculating the LC50 and the associated 95% confidence interval.

2. The program begins with a request for the following initial input (see Figure 10):
   a. Desired output of abbreviated (A) or full (F) output?
   b. Output designation (P = printer, D = disk file).
   c. Title for the output.
   d. Control data.
   c. The number of exposure concentrations
   d. Data for each toxicant concentration

3. The program output includes the following (see Figure 11):
   a. A table of the observed proportion responding, and the proportion responding adjusted for controls.
   b. The calculated chi-squared 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.
   c. The estimated LC50 and 95% confidence limits.
   d. A plot of the fitted regression line with observed data overlaid on the plot.

4. The results of the data analysis for this example are as follows:
   a. The observed proportion mortalities were not adjusted for mortality in the control.
   b. The test for heterogeneity was not significant (the calculated Chi-square was less than the tabular value), thus the Probit Method appears to be appropriate for this data.
   c. The estimate of the LC50 is 22.9% with a 95% confidence interval of (18.8%, 27.8%).

11.3 DETERMINATION OF NO-OBSERVED-ADVERSE-EFFECT CONCENTRATION (NOAEC) FROM MULTI-CONCENTRATION TESTS, AND DETERMINATION OF PASS OR FAIL (PASS/FAIL) FOR SINGLE-CONCENTRATION (PAIRED) TESTS

11.3.1 Determination of the No-Observed-Adverse-Effect Concentration (NOAEC), for multi-concentration toxicity tests, and pass or fail (Pass/Fail) for single-concentration toxicity tests is accomplished using hypothesis testing. The NOAEC is the lowest concentration at which survival is not significantly different from the control. In Pass/Fail tests, the objective is to determine if the survival in the single treatment (effluent or receiving water) is significantly different from the control survival.

11.3.2 The first step in these analyses is to transform the responses, expressed as the proportion surviving, by the arc-sine-square-root transformation (Figures 12 and 13). The arc-sine-square-root transformation is commonly used on proportionality data to stabilize the variance and satisfy the normality requirement. Shapiro Wilk's test may be used to test the normality assumption.

11.3.3 If the data do not meet the assumption of normality and there are four or more replicates per group, then the non-parametric test, Wilcoxon Rank Sum Test, can be used to analyze the data.

11.3.4 If the data meet the assumption of normality, the F test for equality of variances is used to test the homogeneity of variance assumption. Failure of the homogeneity of variance assumption leads to the use of a modified t test, where the pooled variance estimate is adjusted for unequal variance, and the degrees of freedom for the test are adjusted.

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


Do you wish abbreviated (A) of full (F) output? A
Output to printer or disk file (P / D)? P
Title ? PROBIT EXAMPLE

Number of responders in the control group = ? 0
Number of exposure concentrations, exclusive of controls ? 5

Input data starting with the lowest exposure concentration

Concentration = ? 6.25
Number responding = ? 0
Number exposed = ? 20

Concentration = ? 12.5
Number responding = ? 3
Number exposed = ? 20

Concentration = ? 25
Number responding = ? 9
Number exposed = ? 20

Concentration = ? 50
Number responding = ? 20
Number exposed = ? 20

Concentration = ? 100
Number responding = ? 20
Number exposed = ? 20

   Number       Conc.    Number Resp.    Number Exposed
   ------       -----    ------------    --------------
      1        6.2500          0              20
      2       12.5000          3              20
      3       25.0000          9              20
      4       50.0000         20              20
      5      100.0000         20              20

Do you wish to modify your data ? n
The control response rate =  0
Do you wish to modify it? n
```

Figure 10. Example of input for computer program for Probit Method.

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

PROBIT EXAMPLE

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
6.2500	20	0	0.000	0.000
12.5000	20	3	0.1500	0.1500
25.0000	20	9	0.4500	0.4500
50.0000	20	20	1.0000	1.0000
100.0000	20	20	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 3.076
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 7.815

PROBIT EXAMPLE

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	Lower 95% Confidence	Upper Limits
LC/EC 1.00	7.924	4.147	10.959
LC/EC 50.00	22.872	18.787	27.846

Figure 11. Example of output for computer program for Probit Method.

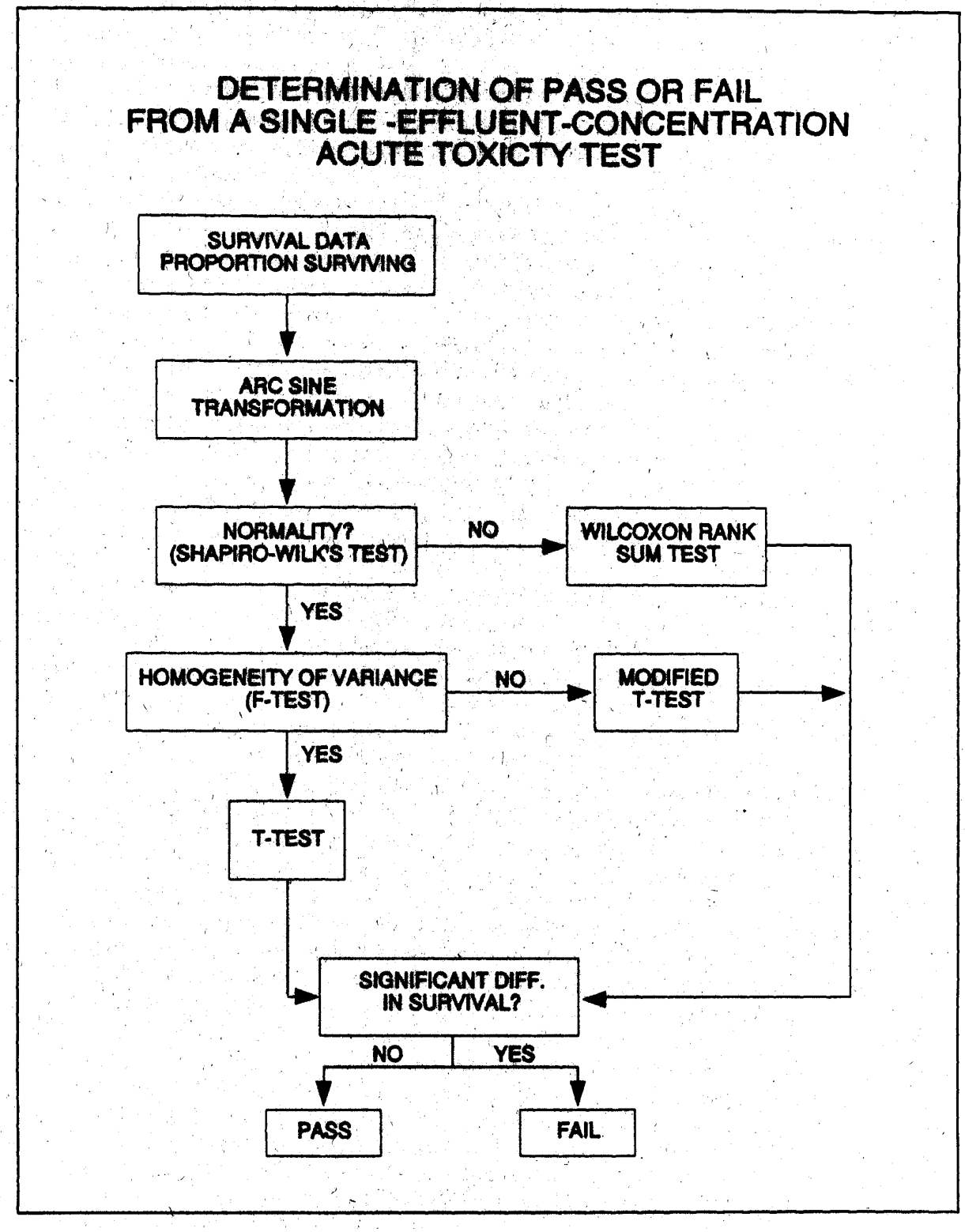

Figure 12. Flowchart for analysis of single-effluent concentration test data.

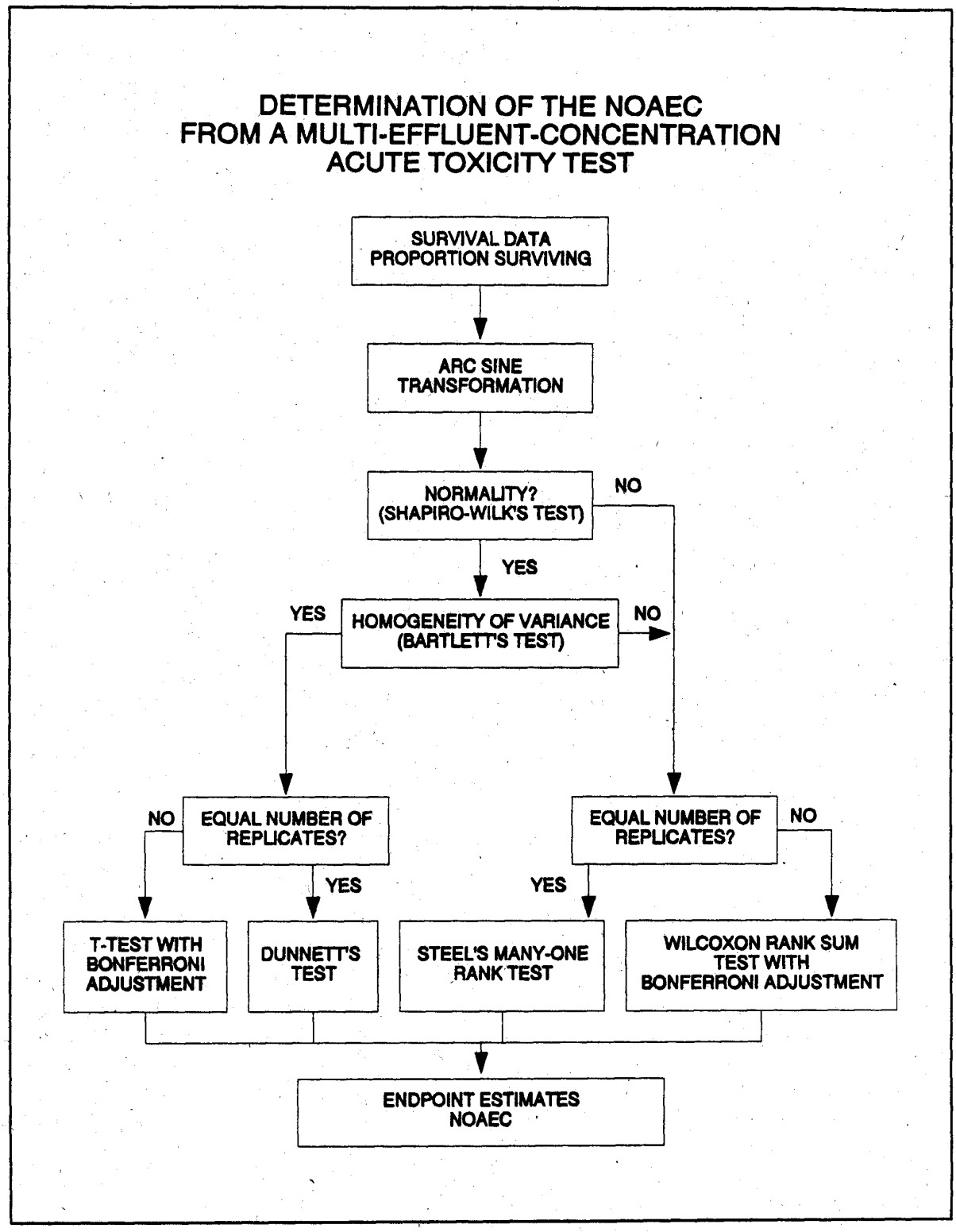

Figure 13. Flowchart for analysis of multi-effluent-concentration test data.

11.3.5 GENERAL PROCEDURE

11.3.5.1 Arc Sine Square Root Transformation

11.3.5.1.1 The arc sine square root transformation consists of determining the angle (in radians) represented by a sine value. In this transformation, the proportion surviving is taken as the sine value, the square root of the sine value is calculated, and the angle (in radians) for the square root of the sine value is determined. Whenever the proportion surviving is 0 or 1, a special modification of the transformation must be used (Bartlett, 1937). Illustrations of the arc sine square root transformation and modification are provided below.

1. Calculate the response proportion (RP) for each replicate within a group, where:

   RP = (number of surviving organisms)/(number exposed)

2. Transform each RP to arc sine, as follows.

   a. For RPs greater than zero or less than one:

   $$Angle(in\ radians) = arc\ sine\sqrt{(RP)}$$

   b. Modification of the arc sine when RP = 0.

   $$Angle(in\ radians) = arc\ sine\sqrt{\frac{1}{4n}}$$

   where n = number animals/treatment rep.

   c. Modification of the arc sine when RP = 1.0.

   $$Angle = 1.5708\ radians - (radians\ for\ RP{=}0)$$

11.3.5.2 Shapiro Wilk's Test

11.3.5.2.1 After the data have been transformed, test the assumption of normality using Shapiro Wilk's test. The test statistic, W, is obtained by dividing the square of an appropriate linear combination of the sample order statistics by the usual symmetric estimate of variance (D). 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, and there must be more than two replicates per concentration for the test to be valid.

1. To calculate W, first center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration.

2. Calculate the denominator, D, of the test statistic:

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

   where: X_i = the ith centered observation

   $\overline{X}$ = the overall mean of the centered observations.

3. Order the centered observations from smallest to largest.

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(i)}$$

where: $X^{(i)}$ denotes the ith ordered observation.

4. From Table 21, 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.

5. Compute the test statistic, W, as follows:

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

11.3.5.2.2 The decision rule for the test is to compare the critical value from Table 22 to the computed W. If the computed value is less than the critical value, conclude that the data are not normally distributed.

11.3.5.3 F Test

11.3.5.3.1 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.

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

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

11.3.5.3.3 Compare the calculated F with the 0.005 level of a tabulated 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 (Snedecor and Cochran, 1980). If the calculated F value is less than or equal to the tabulated F, conclude that the variances of the two groups are equal.

11.3.5.4 T Test

11.3.5.4.1 If the variances for the two groups are found to be statistically equivalent, then the equal variance t test is the appropriate test.

TABLE 21. COEFFICIENTS FOR THE SHAPIRO WILK'S TEST (CONOVER, 1980)

i\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

i\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

i\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 21. COEFFICIENTS FOR THE SHAPIRO WILK'S TEST (CONTINUED)

i\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

i\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

TABLE 22. QUANTILES OF THE SHAPIRO WILK'S TEST STATISTIC[1] (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

11.3.5.4.2 Calculate the following test statistic:

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

where: $\bar{X}_1$ = Mean for the control

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

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

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

11.3.5.4.3 Since we are concerned with a decrease in survival from the control, a one-tailed test is appropriate. Thus, 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.

11.3.5.5 Modified T Test

11.3.5.5.1 If the F test for equality of variance fails, the t test is still a valid test. However, the denominator and the degrees of freedom for the test are modified.

11.3.5.5.2 The t statistic, with the modification for the denominator, is calculated as follows:

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

where: $\bar{X}_1$ = 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

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

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

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

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

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

11.3.5.6 Wilcoxon Rank Sum Test

11.3.5.6.1 If the data fail the test for normality and there are four or more replicates per group, the non-parametric Wilcoxon Rank Sum Test may be used to analyze the data. If less than four replicates were used, a non-parametric alternative is not available.

11.3.5.6.2 The Wilcoxon Rank Sum Test consists of jointly ranking the data and calculating the rank sum for the effluent concentration. The rank sum is then compared to a critical value to determine acceptance or rejection of the null hypothesis.

11.3.5.6.3 To carry out the test, combine the data for the control and the effluent concentration 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. Sum the ranks for the effluent concentration.

11.3.5.6.4 If the survival in the effluent concentration is significantly less than that of the control, the rank sum for the effluent concentration would be lower than the rank sum of the control. Thus, we are only concerned with comparing the rank sum for the effluent concentration with some "minimum" or critical rank sum, at or below which the effluent concentration survival would be considered to be significantly lower than the mortality in the control. For a test at the 5% level of significance, the critical rank sum can be found in Table 23.

TABLE 23. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST FIVE PERCENT CRITICAL LEVEL

No. of Replicates in Control	No. of Replicates per Effluent Concentration							
	3	4	5	6	7	8	9	10
3		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

11.3.6 SINGLE CONCENTRATION TEST

11.3.6.1 Data from an acute effluent toxicity test with *Ceriodaphnia* are provided in Table 24. The proportion surviving in each replicate is transformed by the arc sine square root transformation prior to statistical analysis of the data (Figure 12).

TABLE 24. DATA FROM AN ACUTE SINGLE-CONCENTRATION TOXICITY TEST WITH *CERIODAPHNIA*

		Proportion Surviving	
	Replicate	Control	100% Effluent Concentration
RAW DATA	A	1.00	0.40
	B	1.00	0.30
	C	0.90	0.40
	D	0.90	0.20
ARC SINE TRANSFORMED DATA	A	1.412	0.685
	B	1.412	0.580
	C	1.249	0.685
	D	1.249	0.464
	$\bar{X}$	1.330	0.604
	S^2	0.0088	0.0111

TABLE 25. EXAMPLE OF SHAPIRO WILK'S TEST: CENTERED OBSERVATIONS

	Replicate			
Treatment	A	B	C	D
Control	0.082	0.082	-0.081	-0.081
100% Effluent	0.081	-0.024	0.081	-0.140

11.3.6.2 After the data have been transformed, test the assumption of normality via the Shapiro Wilk's test.

11.3.6.2.1 The first step in 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 25.

11.3.6.2.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{8} (X_i - \bar{X})^2$$

For this set of data, $\bar{X} = 0$ and D = 0.060.

11.3.6.2.3 Order the centered observations from smallest to largest. The ordered observations are listed in Table 26.

11.3.6.2.4 From Table 21, for n = 8 and k = n/2 = 4, obtain the coefficients $a_1, a_2, ..., a_k$. The a_i values are listed in Table 27.

11.3.6.2.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{0.060} \cdot (0.2200)^2 = 0.0807$$

The differences, $X^{(n-i+1)}-X^{(i)}$, are listed in Table 27.

11.3.6.2.6 From Table 22, the critical W value for n = 8 and a significance level of 0.01, is 0.749. Since the calculated W, 0.807, is not less than the critical value the conclusion of the test is that the data are normally distributed.

TABLE 26. EXAMPLE OF SHAPIRO WILK'S TEST: ORDERED OBSERVATIONS

i	$X^{(i)}$
1	-0.140
2	-0.081
3	-0.081
4	-0.024
5	0.081
6	0.081
7	0.082
8	0.082

TABLE 27. EXAMPLE OF SHAPIRO WILK'S TEST: TABLE OF COEFFICIENTS AND DIFFERENCES

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.6052	0.222	$X^{(8)} - X^{(1)}$
2	0.3164	0.163	$X^{(7)} - X^{(2)}$
3	0.1743	0.162	$X^{(6)} - X^{(3)}$
4	0.0561	0.105	$X^{(5)} - X^{(4)}$

11.3.6.3 The F test for equality of variances is used to test the homogeneity of variance assumption.

11.3.6.3.1 From Table 24, obtain the sample variances for the control and the 100% effluent. Since the variability of the 100% effluent is greater than the variability of the control, S^2 for the 100% effluent concentration is placed in the numerator of the F statistic and S^2 for the control is placed in the denominator.

$$F = \frac{0.0111}{0.0088} = 1.2614$$

11.3.6.3.2 There are four replicates for the control and four replicates for the 100% effluent concentration. Thus there are three degrees of freedom for the numerator and the denominator. For a two-tailed test at the 0.01 level of significance, the critical F value is 47.467. The calculated F, 1.2614, is less than the critical F, 47.467, thus the conclusion is that the variances of the control and 100% effluent are equal.

11.3.6.4 The assumptions of normality and homogeneity of variance have been met for this data set. An equal variance t test will be used to compare the mean responses of the control and 100% effluent.

11.3.6.4.1 To perform the t test, obtain the values for X_1, X_2, S_1^2, and S_2^2 from Table 24. Calculate the t statistic as follows:

$$t = \frac{1.330 - 0.604}{0.0997\sqrt{\frac{1}{4} + \frac{1}{4}}}$$

where:

$$S_p = \frac{\sqrt{(4-1)0.0088 + (4-1)(0.0111)}}{4+4-2}$$

11.3.6.4.2 For a one-tailed test at the 0.05 level of significance with 6 degrees of freedom, the critical t value is 1.9432. Since the calculated t, 10.298, is greater than the critical t, the conclusion is that the survival in the 100% effluent concentration is significantly less than the survival in the control.

11.3.6.5 If the data had failed the normality assumption, the appropriate analysis would have been the Wilcoxon Rank Sum Test. To provide an example of this test, the survival data from the t test example will be reanalyzed by the nonparametric procedure.

11.3.6.5.1 The first step in the Wilcoxon Rank Sum Test is to combine the data from the control and the 100% effluent concentration and arrange the values in order of size, from smallest to largest.

11.3.6.5.2 Assign ranks to the ordered observations, a rank of 1 to the smallest, 2 to the next smallest, etc. The combined data with ranks assigned is presented in Table 28.

TABLE 28. EXAMPLE OF WILCOXON'S RANK SUM TEST: ASSIGNING RANKS TO THE CONTROL AND 100% EFFLUENT CONCENTRATIONS

Rank	Proportion Surviving	Control or 100% Effluent
1	0.20	100% EFFLUENT
2	0.30	100% EFFLUENT
3.5	0.40	100% EFFLUENT
3.5	0.40	100% EFFLUENT
5.5	0.90	CONTROL
5.5	0.90	CONTROL
7.5	1.00	CONTROL
7.5	1.00	CONTROL

11.3.6.5.3 Sum the ranks for the 100% effluent concentration.

11.3.6.5.4 For this set of data, the test is for a significant reduction in survival in the 100% effluent concentration as compared to the control. The critical value, from Table 23, for four replicates in each group and a significance level of 0.05 is 11. The rank sum for the 100% effluent concentration is 10 which is less than the critical value of 11. Thus the conclusion is that survival in the effluent concentration is significantly less than the control survival.

11.3.7 MULTI-CONCENTRATION TEST

11.3.7.1 Formal statistical analysis of the survival data is outlined in Figure 13. The response used in the analysis is the proportion of animals surviving in each test or control chamber. Concentrations at which there is no survival in any of the test chambers are excluded from statistical determination of the NOAEC.

11.3.7.2 For the case of equal numbers of replicates across all concentrations and the control, the determination of the NOAEC endpoint is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro Wilk's Test, and Bartlett's Test is used to test for the homogeneity of variance. If either of these tests fail, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOAEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

11.3.7.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t-test with a Bonferroni adjustment. The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

11.3.7.4 Example of Analysis of Survival Data

11.3.7.4.1 This example uses survival data from a fathead minnow test. The proportion surviving in each replicate must first be transformed by the arc sine square root transformation procedure. The raw and transformed data, means and standard deviations of the transformed observations at each toxicant concentration and control are listed in Table 29. A plot of the survival proportions is provided in Figure 14.

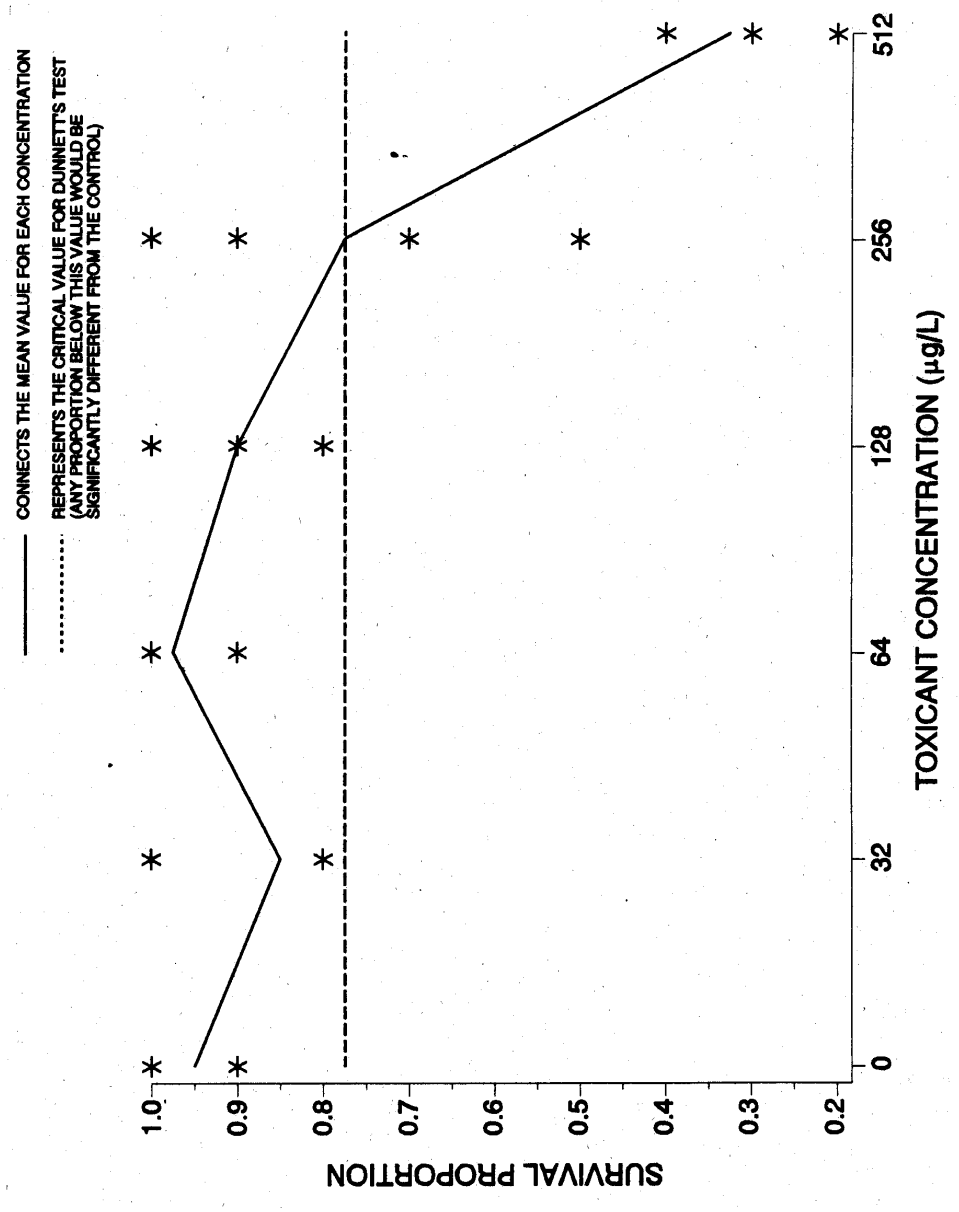

Figure 14. Plot of mean survival proportion data in Table 29.

11.3.7.4.2 Test for Normality

1. 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 summarized in Table 30.

TABLE 29. FATHEAD MINNOW SURVIVAL DATA

			Toxicant Concentration (µg/L)				
	Replicate	Control	32	64	128	256	512
	A	1.0	0.8	0.9	0.9	0.7	0.4
RAW	B	1.0	0.8	1.0	0.9	0.9	0.3
	C	0.9	1.0	1.0	0.8	1.0	0.4
	D	0.9	0.8	1.0	1.0	0.5	0.2
ARC SINE	A	1.412	1.107	1.249	1.249	0.991	0.685
TRANS-	B	1.412	1.107	1.412	1.249	1.249	0.580
FORMED	C	1.249	1.412	1.412	1.107	1.412	0.685
	D	1.249	1.107	1.412	1.412	0.785	0.464
MEAN($\bar{Y}_i$)		1.330	1.183	1.371	1.254	1.109	0.604
S_i^2		0.0088	0.0232	0.0066	0.0155	0.0768	0.0111
i		1	2	3	4	5	6

TABLE 30. CENTERED OBSERVATIONS FOR SHAPIRO WILK'S EXAMPLE

		Toxicant Concentration (µg/L)				
Replicate	Control	32	64	128	256	512
A	0.082	-0.076	-0.122	-0.005	-0.118	0.081
B	0.082	-0.076	0.041	-0.005	0.140	-0.024
C	-0.081	0.229	0.041	-0.147	0.303	0.081
D	-0.081	-0.076	0.041	0.158	-0.324	-0.140

2. Calculate the denominator, D, of the statistic:

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

where: X_i = the ith centered observation
$\bar{X}$ = the overall mean of the centered observations
n = the total number of centered observations

3. For this set of data: n = 24 (number of observations)

$$\bar{X} = \frac{1}{24}(0.000) = 0.000$$

$$D = 0.4265$$

4. Order the centered observations from smallest to largest

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

where: $X^{(i)}$ denotes the ith ordered observation.

The ordered observations for this example are listed in Table 31.

TABLE 31. ORDERED CENTERED OBSERVATIONS FOR THE SHAPIRO WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.324	13	-0.005
2	-0.147	14	0.041
3	-0.140	15	0.041
4	-0.122	16	0.041
5	-0.118	17	0.081
6	-0.081	18	0.081
7	-0.081	19	0.082
8	-0.076	20	0.082
9	-0.076	21	0.140
10	-0.076	22	0.158
11	-0.024	23	0.229
12	-0.005	24	0.303

5. From Table 21, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots a_k$, where k is approximately n/2 if n is even; (n-1)/2 if n is odd. For the data in this example, n=24 and k=12. The a_i values are listed in Table 32.

6. Compute the test statistic, W, as follows:

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

The differences $X^{(n-i+1)}-X^{(i)}$ are listed in Table 32. For the data in this example,

$$W = \frac{1}{0.4265}(0.6444)^2 = 0.974$$

7. The decision rule for this test is to compare W as calculated in #6 to a critical value found in Table 23. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 24 observations is 0.884. Since W = 0.974 is greater than the critical value, conclude that the data are normally distributed.

TABLE 32. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4493	0.627	$X^{(24)} - X^{(1)}$
2	0.3098	0.376	$X^{(23)} - X^{(2)}$
3	0.2554	0.298	$X^{(22)} - X^{(3)}$
4	0.2145	0.262	$X^{(21)} - X^{(4)}$
5	0.1807	0.200	$X^{(20)} - X^{(5)}$
6	0.1512	0.163	$X^{(19)} - X^{(6)}$
7	0.1245	0.162	$X^{(18)} - X^{(7)}$
8	0.0997	0.157	$X^{(17)} - X^{(8)}$
9	0.0764	0.117	$X^{(16)} - X^{(9)}$
10	0.0539	0.117	$X^{(15)} - X^{(10)}$
11	0.0321	0.065	$X^{(14)} - X^{(11)}$
12	0.0107	0.0	$X^{(13)} - X^{(12)}$

11.3.7.4.3 Test for Homogeneity of Variance

1. The test used to examine whether the variation in mean proportion surviving is the same across all toxicant concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i) \ln \overline{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

where: V_i = degrees of freedom for each toxicant concentration and control, $V_i = (n_i - 1)$
n_i = the number of replicates for concentration i.
$1n = \log_e$
i = 1, 2, ..., p where p is the number of concentrations including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

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

2. For the data in this example, (See Table 29) all toxicant concentrations including the control have the same number of replicates ($n_i = 4$ for all i). Thus, $V_i = 3$ for all i.

3. Bartlett's statistic is therefore:

$$B = [(18)1n(0.0236) - 3\sum_{i=1}^{P} 1n(S_i^2)]/1.1296$$

$$= [18(-3.7465) - 3(-24.7516)]/1.1296$$

$$= 6.8178/1.1296$$

$$= 6.036$$

4. B is approximately distributed as chi square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with five degrees of freedom, is 15.086. Since B = 6.036 is less than the critical value of 15.086, conclude that the variances are not different.

11.3.7.4.4 Dunnett's Procedure

1. To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table (Table 33).

TABLE 33. 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 toxicant concentrations including the control
N = total number of observations $n_1 + n_2 ... + n_P$
n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N \qquad \text{Between Sum of Squares}$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N \qquad \text{Total Sum of Squares}$$

$$SSW = SST - SSB \qquad \text{Within Sum of Squares}$$

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"
Y_{ij} = the jth observation for concentration "i" (represents the proportion surviving for toxicant concentration i in test chamber j)

2. For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 4$

$N = 24$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 5.322$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 4.733$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 5.485$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 5.017$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 4.437$
$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} = 2.414$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 27.408$

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

$$= \frac{1}{4}(131.495) - \frac{(27.408)^2}{24} = 1.574$$

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

$$= 33.300 - \frac{(27.408)^2}{24} = 2.000$$

SSW = SST - SSB = 2.000 - 1.574 = 0.4260

S_B2 = SSB/(p - 1) = 1.574/(6 - 1) = 0.3150

S_W^2 = SSW/(N - p) = 0.426/(24 - 6) = 0.024

3. Summarize these calculations in the ANOVA table (Table 34).

TABLE 34. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	DF	Sum of Squares (SS)	Mean Square (MS) (SS/DF)
BETWEEN	5	1.574	0.315
WITHIN	18	0.426	0.024
Total	23	2.002	

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

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

where: $\overline{Y}_i$ = mean proportion surviving for concentration i
$\overline{Y}_1$ = mean proportion surviving for the control
S_W = square root of within mean square
n_1 = number of replicates for control
n_i = number of replicates for concentration i.

5. Table 35 includes the calculated t values for each concentration and control combination. In this example, comparing the 32 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.330 - 1.183)}{[0.155\sqrt{(1/4) + (1/4)}]} = 1.341$$

6. Since the purpose of this test is to detect a significant reduction in proportion surviving, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 36. For an overall alpha level of 0.05, 18 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.41. The mean proportion surviving for concentration "i" is considered significantly less than the mean proportion surviving for the control if t_i is greater than the critical value. Since t is greater than 2.41, the 512 µg/L concentration has significantly lower survival than the control. Hence the NOAEC for survival is 256 µg/L.

TABLE 35. CALCULATED T VALUES

Toxicant Concentration (µg/L)	i	t_i
32	2	1.341
64	3	-0.374
128	4	0.693
256	5	2.016
512	6	6.624

7. To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be detected statistically may be calculated.

$$MSD = d\, S_w \sqrt{(1/n_1) + (1/n)}$$

where: d = the critical value for the Dunnett's procedure
S_W = the square root of the within mean square
n = the common number of replicates at each concentration (this assumes equal replication at each concentration)
n_i = the number of replicates in the control.

8. In this example:

$$MSD = 2.41(0.155)\sqrt{(1/4) + (1/4)}$$

$$= 2.41\ (0.155)(0.707)$$

$$= 0.264$$

9. The MSD (0.264) is in transformed units. To determine the MSD in terms of percent survival, carry out the following conversion.

(1) Subtract the MSD from the transformed control mean.

$$1.330 - 0.264 = 1.066$$

(2) Obtain the untransformed values for the control mean and the difference calculated in 1.

$$[\text{Sine}\ (\ 1.330)\ ]^2 = 0.943$$

$$[\text{Sine}\ (\ 1.066)\ ]^2 = 0.766$$

(3) The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from 2.

$$MSD_u = 0.943 - 0.766 = 0.177$$

10. Therefore, for this set of data, the minimum difference in mean proportion surviving between the control and any toxicant concentration that can be detected as statistically significant is 0.177.

11. This represents a decrease in survival of 19% from the control.

TABLE 36. DUNNETT'S "T" VALUES (MILLER, 1981)

(One-tailed)d^{α}_{k}									
					$\alpha = .05$				
	1	2	3	4	5	6	7	8	9
5	2.02	2.44	2.58	2.85	2.98	3.08	3.16	3.24	3.30
6	1.94	2.34	2.56	2.71	2.83	2.92	3.00	3.07	3.12
7	1.89	2.27	2.48	2.62	2.73	2.82	2.89	2.95	3.01
8	1.86	2.22	2.42	2.55	2.66	2.74	2.81	2.87	2.92
9	1.83	2.18	2.37	2.50	2.60	2.68	2.75	2.81	2.86
10	1.81	2.15	2.34	2.47	2.56	2.64	2.70	2.76	2.81
11	1.80	2.13	2.31	2.44	2.53	2.60	2.67	2.72	2.77
12	1.78	2.11	2.29	2.41	2.50	2.58	2.64	2.69	2.74
13	1.77	2.09	2.27	2.39	2.48	2.55	2.61	2.68	2.71
14	1.76	2.08	2.25	2.37	2.46	2.53	2.59	2.64	2.69
15	1.75	2.07	2.24	2.36	2.44	2.51	2.57	2.62	2.67
16	1.75	2.06	2.23	2.34	2.43	2.50	2.56	2.61	2.65
17	1.74	2.05	2.22	2.33	2.42	2.49	2.54	2.59	2.64
18	1.73	2.04	2.21	2.32	2.41	2.48	2.53	2.58	2.62
19	1.73	2.03	2.20	2.31	2.40	2.47	2.52	2.57	2.61
20	1.72	2.03	2.19	2.30	2.38	2.46	2.51	2.56	2.60
24	1.71	2.01	2.17	2.28	2.36	2.43	2.48	2.53	2.57
30	1.70	1.99	2.15	2.25	2.33	2.40	2.45	2.50	2.54
40	1.68	1.97	2.13	2.23	2.31	2.37	2.42	2.47	2.51
60	1.67	1.95	2.10	2.21	2.28	2.35	2.39	2.44	2.48
120	1.86	1.93	2.08	2.18	2.26	2.32	2.37	2.41	2.45
α	1.64	1.92	2.06	2.16	2.23	2.29	2.34	2.33	2.42

TABLE 36. DUNNETT'S "T" VALUES (MILLER, 1981) (CONTINUED)

(One-tailed)d^{α}_{k}									
					$\alpha = 0.1$				
	1	2	3	4	5	6	7	8	9
5	3.37	3.90	4.21	4.43	4.50	4.73	4.85	4.94	5.03
6	3.14	3.61	4.88	4.07	4.21	4.33	4.43	4.51	4.39
7	3.00	3.42	3.56	3.83	3.96	4.07	4.15	4.23	4.30
8	2.90	3.20	3.51	3.67	3.79	3.88	3.96	4.03	4.09
9	2.82	3.19	3.40	3.55	3.64	3.75	3.82	3.89	3.94
10	2.76	3.11	3.31	3.45	3.56	3.64	3.71	3.78	3.83
11	2.72	3.06	3.25	3.38	3.46	3.56	3.63	3.69	3.74
12	2.68	3.01	3.19	3.32	3.42	3.50	3.56	3.62	3.67
13	2.65	2.97	3.15	3.27	3.37	3.44	3.91	3.56	3.61
14	2.62	2.94	3.11	3.23	3.32	3.40	3.46	3.51	3.56
15	2.60	2.91	3.08	3.20	3.29	3.36	3.42	3.47	3.52
16	2.58	2.38	3.05	3.17	3.28	3.33	3.39	3.44	3.48
17	2.57	2.86	3.03	3.14	3.23	3.30	3.36	3.41	3.45
18	2.55	2.84	3.01	3.12	3.21	3.27	3.33	3.38	3.42
19	2.54	2.83	2.99	3.10	3.18	3.25	3.31	3.36	3.40
20	2.53	2.81	2.97	3.08	3.17	3.23	3.29	3.34	3.38
24	2.40	2.77	2.92	3.03	3.11	3.17	3.22	3.27	3.31
30	2.46	2.72	2.87	2.97	3.05	3.11	3.16	3.21	3.24
40	2.42	2.68	2.82	2.92	2.99	3.06	3.10	3.14	3.18
60	2.39	2.64	2.78	2.87	2.94	3.08	3.04	3.06	3.12
120	2.36	2.60	2.73	2.82	2.90	2.94	2.90	3.03	3.06
α	2.33	2.56	2.68	2.77	2.34	2.90	2.83	2.97	3.00

SECTION 12

REPORT PREPARATION AND TEST REVIEW

12.1 REPORT PREPARATION

The following general format and content are recommended for the report:

12.1.1 INTRODUCTION

1. Permit number
2. Toxicity testing requirements of permit
3. Plant location
4. Name of receiving water body
5. Contractor (if contracted)
    a. Name of firm
    b. Phone number
    c. Address
6. Objective of test

12.1.2 PLANT OPERATIONS

1. Product(s)
2. Raw materials
3. Operating schedule
4. Description of waste treatment
5. Schematic of waste treatment
6. Retention time (if applicable)
7. Volume of discharge (MGD, CFS, GPM)
8. Design flow of treatment facility at time of sampling

12.1.3 SOURCE OF EFFLUENT, RECEIVING WATER, AND DILUTION WATER

1. Effluent Samples
    a. Sampling point (including latitude and longitude)
    b. Sample collection method
    c. Collection dates and times
    d. Mean daily discharge on sample collection date
    e. Lapsed time from sample collection to delivery
    f. Sample temperature when received at the laboratory
    g. Physical and chemical data

2. Receiving Water Samples
    a. Sampling point (including latitude and longitude)
    b. Sample collection method
    c. Collection dates and times
    d. Streamflow (at time of sampling)
    e. Lapsed time from sample collection to delivery
    f. Sample temperature when received at the laboratory
    g. Physical and chemical data

3. Dilution Water Samples
    a. Source
    b. Collection date(s) and time(s) (where applicable)
    c. Pretreatment
    d. Physical and chemical characteristics (pH, hardness, salinity, etc.)

12.1.4 TEST CONDITIONS

1. Toxicity test method used (title, number, source)
2. Endpoint(s) of test
3. Deviations from reference method, if any, and reason(s)
4. Date and time test started
5. Date and time test terminated
6. Type and volume of test chambers
7. Volume of solution used per chamber
8. Number of organisms per test chamber
9. Number of replicate test chambers per treatment
10. Feeding frequency, and amount and type of food
11. Acclimation temperature of test organisms (mean and range)
12. Test temperature (mean and range)

12.1.5 TEST ORGANISMS

1. Scientific name
2. Age
3. Life stage
4. Mean length and weight (where applicable)
5. Source
6. Diseases and treatment (where applicable)

12.1.6 QUALITY ASSURANCE

1. Reference toxicant used routinely; source; date received; lot no.
2. Date and time of most recent reference toxicant test; test results and current cusum chart
3. Dilution water used in reference toxicant test
4. Physical and chemical methods used

12.1.7 RESULTS

1. Provide raw toxicity data in tabular form, including daily records of affected organisms in each concentration (including controls) and replicate, and in graphical form (plots of toxicity data)
2. Provide table of endpoints: LC50, NOAEC, Pass/Fail (as required in the applicable NPDES permit)
3. Indicate statistical methods used to calculate endpoints
4. Provide summary table of physical and chemical data
5. Tabulate QA data

12.1.8 CONCLUSIONS AND RECOMMENDATIONS

1. Relationship between test endpoints and permit limits.
2. Action to be taken.

12.2 TEST REVIEW

12.2.1 Test review is an important part of an overall quality assurance program (Section 4) and is necessary for ensuring that all test results are reported accurately. Test review should be conducted on each test by both the testing laboratory and the regulatory authority.

12.2.2 SAMPLING AND HANDLING

12.2.2.1 The collection and handling of samples are reviewed to verify that the sampling and handling procedures given in Section 8 were followed. Chain-of-custody forms are reviewed to verify that samples were tested within allowable sample holding times (Subsection 8.5.4). Any deviations from the procedures given in Section 8 should be documented and described in the data report (Subsection 12.1).

12.2.3 TEST ACCEPTABILITY CRITERIA

12.2.3.1 Test data are reviewed to verify that test acceptability criteria (TAC) requirements for a valid test have been met. Any test not meeting the minimum test acceptability criteria is considered invalid. All invalid tests must be repeated with a newly collected sample.

12.2.4 TEST CONDITIONS

12.2.4.1 Test conditions are reviewed and compared to the specifications listed in the summary of test condition tables provided for each method. Physical and chemical measurements taken during the test (e.g., temperature, pH, and DO) also are reviewed and compared to specified ranges. Any deviations from specifications should be documented and described in the data report (Subsection 12.1).

12.2.4.2 The summary of test condition tables presented for each method identify test conditions as required or recommended. For WET test data submitted under NPDES permits, all required test conditions must be met or the test is considered invalid and must be repeated with a newly collected sample. Deviations from recommended test conditions must be evaluated on a case-by-case basis to determine the validity of test results. Deviations from recommended test conditions may or may not invalidate a test result depending on the degree of the departure and the objective of the test. The reviewer should consider the degree of the deviation and the potential or observed impact of the deviation on the test result before rejecting or accepting a test result as valid. For example, if dissolved oxygen is measured below 4.0 mg/L in one test chamber, the reviewer should consider whether any observed mortality in that test chamber corresponded with the drop in dissolved oxygen.

12.2.4.3 Whereas slight deviations in test conditions may not invalidate an individual test result, test condition deviations that continue to occur frequently in a given laboratory may indicate the need for improved quality control in that laboratory.

12.2.5 STATISTICAL METHODS

12.2.5.1 The statistical methods used for analyzing test data are reviewed to verify that the recommended flowcharts for statistical analysis were followed. Any deviation from the recommended flowcharts for selection of statistical methods should be noted in the data report. Statistical methods other than those recommended in the statistical flowcharts may be appropriate (see Subsection 11.1.4), however, the laboratory must document the use of and provide the rationale for the use of any alternate statistical method. In all cases (flowchart recommended methods or alternate methods), reviewers should verify that the necessary assumptions are met for the statistical method used.

12.2.6 CONCENTRATION-RESPONSE RELATIONSHIPS

12.2.6.1 The concept of a concentration-response, or more classically, a dose-response relationship is "the most fundamental and pervasive one in toxicology" (Casarett and Doull, 1975). This concept assumes that there is a causal relationship between the dose of a toxicant (or concentration for toxicants in solution) and a measured response. A response may be any measurable biochemical or biological parameter that is correlated with exposure to the toxicant. The classical concentration-response relationship is depicted as a sigmoidal shaped curve, however, the particular shape of the concentration-response curve may differ for each coupled toxicant and response pair. In general, more severe responses (such as acute effects) occur at higher concentrations of the toxicant, and less severe responses (such as chronic effects) occur at lower concentrations. A single toxicant also may produce multiple responses, each characterized by a concentration-response relationship. A corollary of the concentration-response concept is that every toxicant should exhibit a concentration-response relationship, given that the appropriate response is measured and given that the concentration range evaluated is appropriate. Use of this concept can be helpful in determining whether an effluent possesses toxicity and in identifying anomalous test results.

12.2.6.2 The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. USEPA (2000a) provides guidance on evaluating concentration-response relationships to assist in determining the validity of WET test results. All WET test results (from multi-concentration tests) reported under the NPDES program should be reviewed and reported according to USEPA guidance on the evaluation of concentration-response relationships (USEPA, 2000a). This guidance provides review steps for 10 different concentration-response patterns that may be encountered in WET test data. Based on the review, the guidance provides one of three determinations: that calculated effect concentrations are reliable and should be reported, that calculated effect concentrations are anomalous and should be explained, or that the test was inconclusive and the test should be repeated with a newly collected sample. It should be noted that the determination of a valid concentration-response relationship is not always clear cut. Data from some tests may suggest consultation with professional toxicologists and/or regulatory officials. Tests that exhibit unexpected concentration-response relationships also may indicate a need for further investigation and possible retesting.

12.2.7 REFERENCE TOXICANT TESTING

12.2.7.1 Test review of a given effluent or receiving water test should include review of the associated reference toxicant test and current control chart. Reference toxicant testing and control charting is required for documenting the quality of test organisms (Subsection 4.7) and ongoing laboratory performance (Subsection 4.15). The reviewer should verify that a quality control reference toxicant test was conducted according to the specified frequency required by the permitting authority or recommended by the method (e.g., monthly). The test acceptability criteria, test conditions, concentration-response relationship, and test sensitivity of the reference toxicant test are reviewed to verify that the reference toxicant test conducted was a valid test. The results of the reference toxicant test are then plotted on a control chart (see Subsection 4.15) and compared to the current control chart limits (± 2 standard deviations).

12.2.7.2 Reference toxicant tests that fall outside of recommended control chart limits are evaluated to determine the validity of associated effluent and receiving water tests (see Subsection 4.15). An out of control reference toxicant test result does not necessarily invalidate associated test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increasing test organism sensitivity or toward decreasing test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test. More frequent and/or concurrent reference toxicant testing may be advantageous if recent problems (e.g., invalid tests, reference toxicant test results outside of control chart limits, reduced health of organism cultures, or increased within-test variability) have been identified in testing.

12.2.8 TEST VARIABILITY

12.2.8.1 The within-test variability of individual tests should be reviewed. Excessive within-test variability may invalidate a test result and warrant retesting. For evaluating within-test variability, reviewers should consult EPA guidance on upper and lower percent minimum significant difference (PMSD) bounds (USEPA, 2000b).

12.2.8.2 USEPA guidance on WET variability recommends incorporating upper and lower bounds using the PMSD to control and minimize within-test method variability and increase test sensitivity (USEPA, 2000b). The minimum significant difference (MSD) is the smallest difference between the control and another test treatment that can be determined as statistically significant in a given test, and the PMSD is the MSD represented as a percentage of the control response. The equation and examples of MSD calculations are shown in Subsection 11.3.7.4.4.

12.2.8.3 To assist in reviewing within-test variability, EPA recommends maintaining control charts of PMSDs calculated for successive effluent tests (USEPA, 2000b). A control chart of PMSD values characterizes the range of variability observed within a given laboratory, and allows comparison of individual test PMSDs with the laboratory's typical range of variability. Control charts of other variability and test performance measures, such as the MSD, standard deviation or CV of control responses, or average control response, also may be useful for reviewing tests and minimizing variability.

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