United States
Environmental Protection
Agency

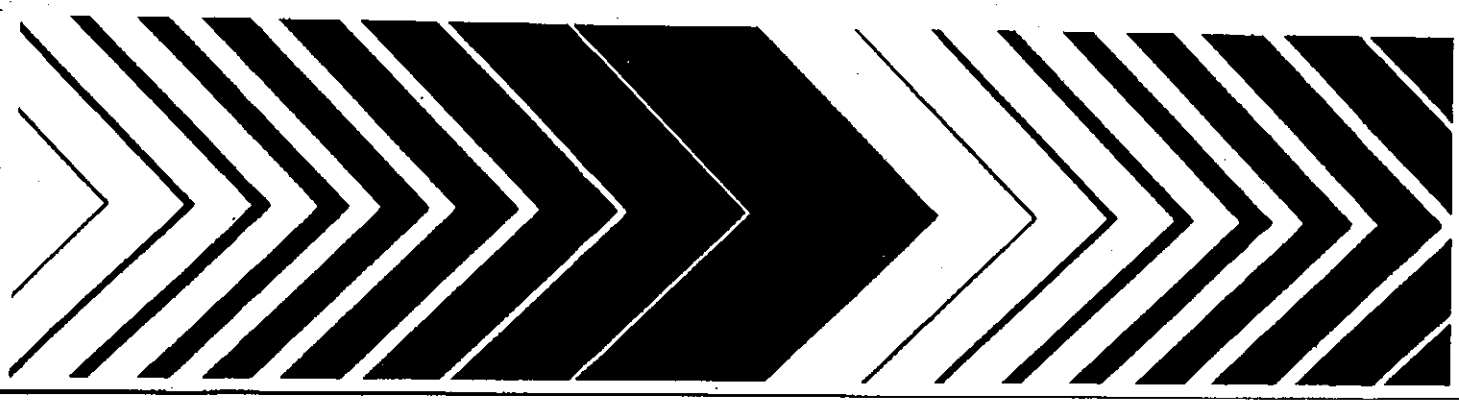
Office of Research and
Development
Washington DC 20460

EPA/600/4-90/027F
August 1993

EPA

Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms

Fourth Edition



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ST CONDITIONS AND TEST ACCEPTABILITY CRITERIA
E, *MENIDIA BERYLLINA*, *M. MENIDIA*, AND *M.*
CUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING
NUED)

---

n: None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min

Uncontaminated source of seawater, deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, modified GP2, or equivalent) prepared with MILLI-Q® or equivalent deionized water (see Section 7, Dilution Water): or receiving water:
1-32‰ ± 10% for *M. beryllina*;
15-32‰ ± 10% for *M. menidia*; and
M. peninsulae

Effluents: Minimum of five effluent concentrations and a control

Receiving Waters: 100% receiving water and a control

Effluents: ≥0.5 dilution series

Receiving Waters: None, or ≥0.5 dilution series

Effluents: Mortality (LC50 or NOAEC)

Receiving Waters: Mortality (Significant difference from control)

Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period.

d: 1 L for effluents

TABLE 17. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SILVERSIDE, *MENIDIA BERYLLINA*, *M. MENIDIA*, AND *M. PENINSULAE*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	Uncontaminated source of seawater, deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, modified GP2, or equivalent) prepared with MILLI-Q® or equivalent deionized water (see Section 7, Dilution Water): or receiving water: 1-32‰ ± 10% for *M. beryllina*; 15-32‰ ± 10% for *M. menidia*; and *M. peninsulae*
18. Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19. Dilution series:	Effluents: ≥0.5 dilution series Receiving Waters: None, or ≥0.5 dilution series
20. Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (Significant difference from control)
21. Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period.
22. Sample volume required:	1 L for effluents 2 L for receiving waters
23. Test acceptability criterion:	90% or greater survival in controls

SECTION 10

TEST DATA

10.1 BIOLOGICAL DATA

10.1.1 Death is the "effect" used for determining toxicity to aquatic organisms in acute toxicity tests.

10.1.2 Death is not easily determined for some organisms. The criteria usually employed in establishing death are: (1) no movement of gills or appendages; and (2) no reaction to gentle prodding.

10.1.3 The death of some organisms, such as mysids and larval fish, is easily detected because of a change in appearance from transparent or translucent to opaque. General observations of appearance and behavior, such as erratic swimming, loss of reflex, discoloration, excessive mucus production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism, should also be noted in the daily record.

10.1.4 The test chambers should be checked for early mortality during the first few hours of the test. The number of surviving organisms in each test chamber is recorded at the end of each 24-h period (Figure 4). When recognizable, dead organisms should be removed during each observation period.

10.1.5 The species, source, and age of the test organisms should be recorded.

10.2 CHEMICAL AND PHYSICAL DATA

10.2.1 In static tests, at a minimum pH, salinity or conductivity, and total residual chlorine are measured in the highest concentration of test solution and in the dilution water at the beginning of the test, at test solution renewal, and at test termination. DO, pH, and temperature are measured in the control and all test concentrations at the beginning of the test, daily thereafter, and at test termination.

10.2.1.1 It is recommended that total alkalinity and total hardness also be measured in the control and highest effluent concentration at the beginning of the test and at test solution renewal.

10.2.1.2 Total ammonia is measured in samples where toxicity may be contributed by unionized ammonia (where total ammonia might be $\geq$5 mg/L).

10.2.1.3 The DO should be monitored closely (every 2 h) for the first 4 to 8 h, to guard against rapid DO depletion, and is measured daily thereafter in all effluent concentrations in which there are surviving organisms, and at test termination. It is recommended that test solution DO be recorded continuously in the test chamber at the highest test solution concentration or in a surrogate vessel at a comparable test solution concentration and containing the standard complement of test organisms.

10.2.1.4 At a minimum, test solution temperature is measured at the beginning of the test, and daily thereafter. Temperature measurements are made by placing thermometers or other temperature sensing devices directly in test solutions or in a comparable volumes of water in chambers positioned in several locations among the test vessels to determine test solution temperatures. It is recommended that test solution temperature be recorded continuously in at least one test chamber or in a comparable volume of water in a surrogate vessel which is comparable to the test chambers.

10.2.2 In flow-through tests, at a minimum pH, salinity or conductivity, total alkalinity, total hardness, and total residual chlorine are measured daily in the highest effluent concentration. DO and temperature are measured at the beginning of the test, daily thereafter in the control and all test concentrations, and at test termination.

10.2.3 The measurement of specific conductance is recommended because it is a very useful parameter in detecting transient fluctuations in the chemical characteristics of effluents, and will indicate errors in test dilutions.

10.2.4 Where acute toxicity test methods are utilized to determine permit limits for toxic chemicals, at a minimum, the concentration of the test material must be measured in each test concentration at test initiation, daily thereafter, and at test termination.

10.2.5 Methods used for chemical analysis should be those specified for Section 304(h) of the CWA (USEPA, 1993c). For salinity measurements, a refractometer may be used if calibrated with a sample of known salinity.

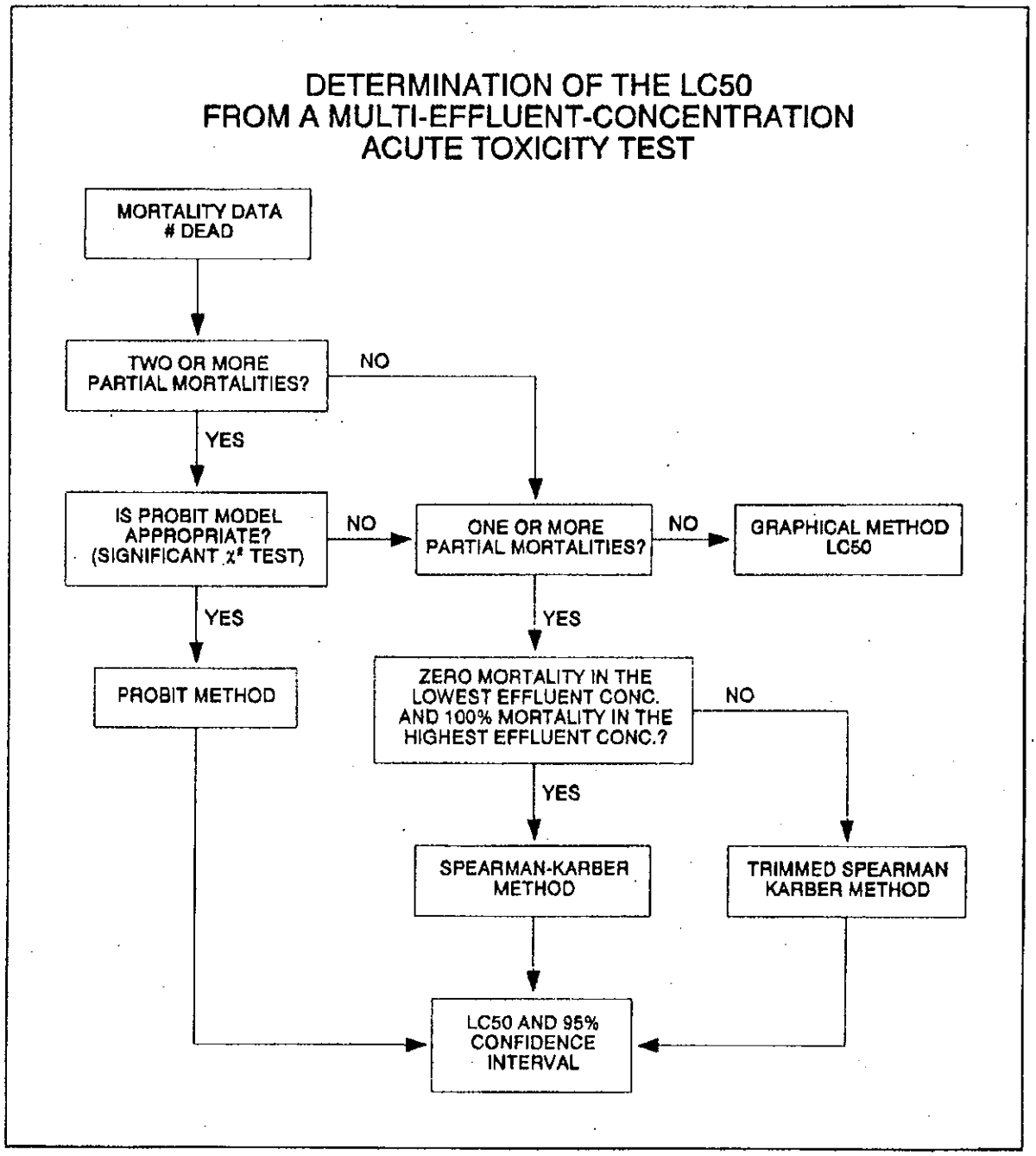

Figure 6. Flowchart for determination of the LC50 for multi-effluent-concentration acute toxicity tests.

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%.

11.2.2.3 General Procedure

1. Let $p_0, p_1, \ldots, p_k$ 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 $p_0 \leq \ldots \leq p_k$. The smoothing replaces any adjacent p_i's that do not conform to $p_0 \leq \ldots \leq p_k$, 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_0^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 18 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_0^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.

TABLE 18. 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

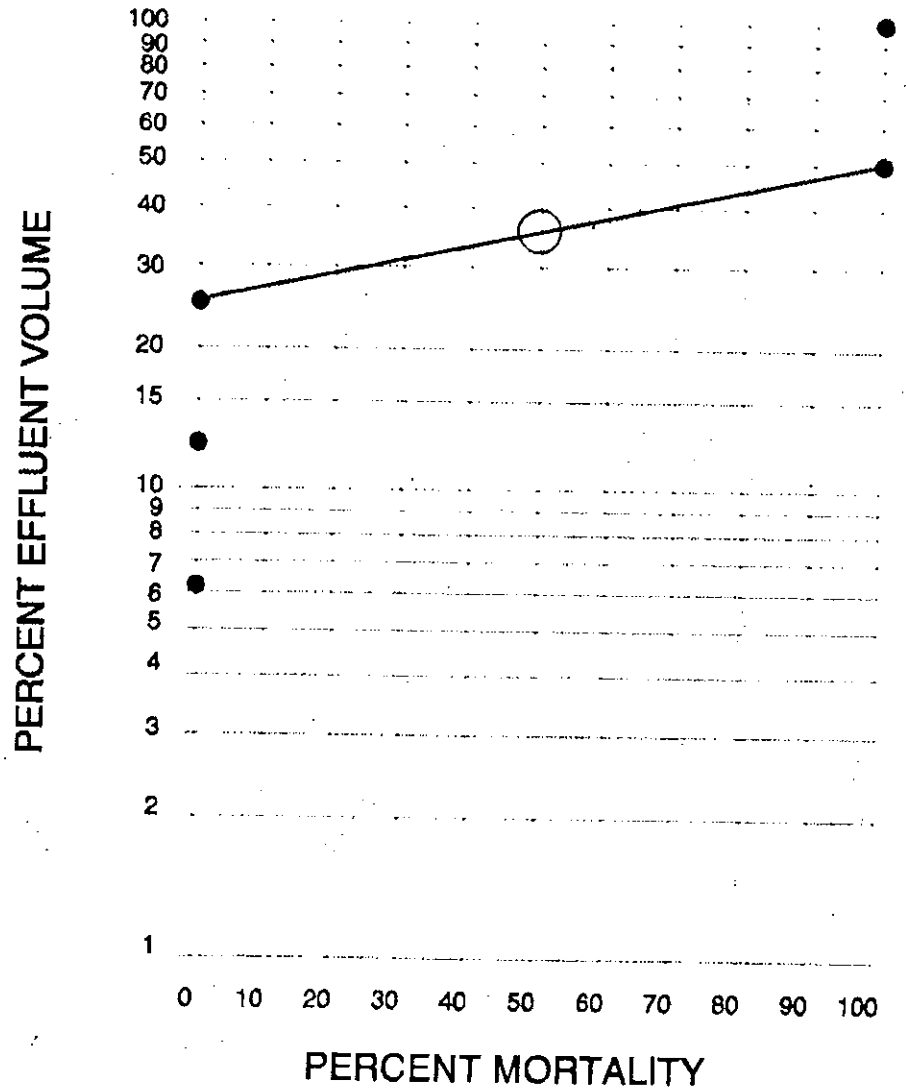

Figure 7. Plotted data and fitted line for graphical method, using all-or-nothing data.

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:

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

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 machine-readable, compiled, version of a computer program which estimates the LC50 and associated 95% confidence interval using the Trimmed Spearman-Karber Method can be obtained from EMSL-Cincinnati by sending a diskette with a written request to the Ecological Monitoring Research Division, Environmental Monitoring Systems Laboratory, at 26 W. Martin Luther King Drive, Cincinnati, OH 45268.

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 18 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.

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:

      $$\text{trim} = \max\{0.00, 0.204\} = 0.204.$$

   c. The estimate of the LC50 is 77.3%. The 95% confidence interval could not be calculated for the data set.

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.

```
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.

```
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.

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 Ecological Monitoring Research Division, Environmental Monitoring Systems Laboratory, 26 W. Martin Luther King Drive, Cincinnati, OH 45268.

11.2.5.4 Example Using the Computer Program

1. Data from Table 18 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.
   e. The number of exposure concentrations
   f. Data for each toxicant concentration.

3. The program output for the abbreviated option 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.

```
        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 Limits	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.

c. The estimated LC1 and 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 responding 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 highest 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 proportional data to stabilize the variance and satisfy the normality and homogeneity of variance requirements. 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 nonparametric 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.

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, 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 replicate.

   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

11.2.5.2.1 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.

TABLES

TABLES (CONTINUED)

ACKNOWLEDGMENTS

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1. Sections 1-10 and 12: General Guidelines
   Margarete A. Heber
   James M. Lazorchak
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   George E. Morrison
   William H. Peltier
   Cornelius I. Weber

2. Section 11: Data Analysis
   Florence Fulk
   Laura Gast
   Cathy Poore

3. Appendices
   A.1. *Ceriodaphnia dubia* - Philip A. Lewis and James M. Lazorchak
   A.2. *Daphnia (D. magna and D. pulex)* - Philip A. Lewis and James M. Lazorchak
   A.3. Mysids (*Mysidopsis bahia*) - Stephan H. Ward
   A.4. Brine Shrimp (*Artemia salina*) - Philip A. Lewis and David A. Bengtson
   A.5. Fathead Minnow, (*Pimephales promelas*) - Donald J. Klemm, Quentin H. Pickering, and Mark E. Smith
   A.6. Rainbow Trout, (*Oncorhynchus mykiss*) and Brook Trout, (*Salvelinius fontinalis*) - Donald J. Klemm
   A.7. Sheepshead Minnow, *(Cyprinodon variegatus)* - Donald J. Klemm
   A.8. Silversides: Inland Silverside *(Mendia beryllina)*, Atlantic Silverside *(M. menidia)*, and Tidewater Silverside *(M. peninsulae)* - Douglas P. Middaugh and Donald J. Klemm
   B. Supplemental List of Acute Toxicity Test Species - Margarete A. Heber
   C. Diluter Systems - William H. Peltier
   D. Plans for Mobile Toxicity Test Laboratory - William H. Peltier
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DISCLAIMER

This document has been reviewed by the Environmental Monitoring Systems Laboratory-Cincinnati (EMSL-Cincinnati), U. S. Environmental Protection Agency (USEPA), and approved for publication. The mention of trade names or commercial products does not constitute endorsement or recommendation for use. The results of data analyses by computer programs described in the section on data analysis were verified using data commonly obtained from effluent toxicity tests. However, these computer programs may not be applicable to all data, and the USEPA assumes no responsibility for their use.

FOREWORD

Environmental measurements are required to determine the quality of ambient waters and the character of waste effluents. The Environmental Monitoring Systems Laboratory-Cincinnati (EMSL-Cincinnati) conducts research to:

- Develop and evaluate analytical methods to identify and measure the concentration of chemical pollutants in drinking waters, surface waters, groundwaters, wastewaters, terrestrial ecosystems, sediments, sludges, and solid wastes.

- Investigate methods for the identification and measurement of viruses, bacteria and other microbiological organisms in drinking waters, surface waters, groundwaters, wastewaters, terrestrial ecosystems, sediments, sludges, and solid wastes to determine the response of aquatic organisms.

- Develop and operate a quality assurance program to support the achievement of data quality objectives in measurements of pollutants in drinking water, surface water, groundwater, wastewater, terrestrial ecosystems, sediment, sludges, and solid waste.

- Develop methods and models to detect and quantify exposures and responses in aquatic and terrestrial organisms to environmental stressors and to correlate the exposure with responses on chemical and biological indicators.

The Federal Water Pollution Control Act Amendments of 1972 (PL 92-500), the Clean Water Act (CWA) of 1977 (PL 95-217), and the Water Quality Act of 1987 (PL 100-4) explicitly state that it is the national policy that the discharge of toxic substances in toxic amounts be prohibited. The detection of chronically toxic effects, therefore, plays an important role in identifying and controlling toxic discharges to surface waters. This manual is a fourth edition of the acute toxicity test manual for effluents first published (EPA/600/4-78/012) by EMSL-Cincinnati in 1978. It provides updated methods for estimating the acute toxicity of effluents and receiving waters to freshwater organisms for use by the U.S. Environmental Protection Agency (USEPA) regional and state programs, and National Pollutant Discharge Elimination System (NPDES) permittees.

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ABSTRACT

This manual describes methods for measuring the acute toxicity of effluents to freshwater, estuarine, and marine macroinvertebrates and fish. The methods include single and multiple concentration static non-renewal, static-renewal, and flow-through toxicity tests for effluents and receiving waters. Also included are guidelines on laboratory safety; quality assurance; facilities and equipment; test species selection and handling; dilution water; effluent and receiving water sample collection, preservation, shipping, and holding; test conditions; toxicity test data analysis; report preparation; organism culturing; and dilutor and mobile laboratory construction.

PREFACE

This manual represents the fourth edition of the Agency's general purpose effluent acute toxicity test manual initially published by EMSL-Cincinnati in January, 1978. This edition reflects changes recommended by the Toxicity Assessment Subcommittee of EPA's Office of Research and Development Biological Advisory Committee, USEPA headquarters program offices and regional staff, other Federal agencies, protection groups, trade associations, major industries, consulting firms, academic institutions engaged in aquatic toxicology research, and other interested parties in the private sector.

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Many, useful public comments on the third edition of the acute toxicity test methods (EPA/600/4-85/013) were received in response to the proposed rule, published in the Federal Register, December 4, 1989 [FR 54(231):50216-50224], regarding the Agency's intent to include the acute toxicity tests in Table IA, 40 CFR Part 136. These comments were considered in the preparation of the fourth edition of the manual, and are included in the Public Docket for the rulemaking, located at room 2904, EPA Headquarters, Washington, D.C.

SECTION 1

INTRODUCTION

1.1 This manual describes acute toxicity tests for use in the National Pollutant Discharge Elimination System (NPDES) Permits Program to identify effluents and receiving waters containing toxic materials in acutely toxic concentrations. The methods included in this manual are referenced in Table IA, 40 CFR Part 136 regulations and, therefore, constitute approved methods for acute toxicity tests. They are also suitable for determining the toxicity of specific compounds contained in discharges. The tests may be conducted in a central laboratory or on-site, by the regulatory agency or the permittee.

1.2 The data are used for NPDES permits development and to determine compliance with permit toxicity limits. Data can also be used to predict potential acute and chronic toxicity in the receiving water, based on the LC50 and appropriate dilution, application, and persistence factors. The tests are performed as a part of self-monitoring permit requirements, compliance biomonitoring inspections, toxics sampling inspections, and special investigations. Data from acute toxicity tests performed as part of permit requirements are evaluated during compliance evaluation inspections and performance audit inspections.

1.3 Modifications of these tests are also used in toxicity reduction evaluations and toxicity identification evaluations to identify the toxic components of an effluent, to aid in the development and implementation of toxicity reduction plans, and to compare and control the effectiveness of various treatment technologies for a given type of industry, irrespective of the receiving water (USEPA, 1988a; USEPA, 1988b; USEPA, 1989a; USEPA, 1989b; USEPA, 1991a).

1.4 This methods manual serves as a companion to the short-term chronic toxicity test methods manuals for freshwater and marine organisms (USEPA, 1993a; USEPA, 1993b), the NPDES compliance inspection manual (1988c), and the manual for evaluation of laboratories performing aquatic toxicity tests (USEPA, 1991b).

1.5 Guidance for the implementation of toxicity tests in the NPDES program is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991c).

1.6 The use of any test species or test conditions other than those described in Tables 11-17 in this manual and referenced in Table 1A, 40 CFR 136.3, shall be considered a major modification to the method and subject to application and approval of alternate test procedures under 40 CFR 136.4 and 40 CFR 136.5.

1.7 These methods are restricted to use by, or under the supervision of, analysts experienced in the use or conduct of, and interpretation of data from, aquatic toxicity tests. Each analyst must demonstrate the ability to generate acceptable test results with the methods using the procedures described in this methods manual.

1.8 This manual was prepared in the established EMSL-Cincinnati format (USEPA, 1983a).

SECTION 2

TYPES OF TESTS

2.1 The selection of the test type will depend on the NPDES permit requirements, the objectives of the test, the available resources, the requirements of the test organisms, and effluent characteristics such as fluctuations in effluent toxicity.

2.2 Effluent acute toxicity is generally measured using a multi-concentration, or definitive test, consisting of a control and a minimum of five effluent concentrations. The tests are designed to provide dose-response information, expressed as the percent effluent concentration that is lethal to 50% of the test organisms (LC50) within the prescribed period of time (24-96h), or the highest effluent concentration in which survival is not statistically significantly different from the control.

2.3 Use of pass/fail tests consisting of a single effluent concentration (e.g., the receiving water concentration or RWC) and a control is not recommended. If the NPDES permit has a whole effluent toxicity limit for acute toxicity at the RWC, it is prudent to use that permit limit as the midpoint of a series of five effluent concentrations. This will ensure that there is sufficient information on the dose-response relationship. For example, the effluent concentrations utilized in a test may be: (1) 100% effluent, (2) (RWC + 100)/2, (3) RWC, (4) RWC/2, and (5) RWC/4. More specifically, if the RWC = 50%, the effluent concentrations used in the toxicity test would be 100%, 75%, 50%, 25%, and 12.5%.

2.4 Receiving (ambient) water toxicity tests commonly employ two treatments, a control and the undiluted receiving water, but may also consist of a series of receiving water dilutions.

2.5 A negative result from an acute toxicity test does not preclude the presence of chronic toxicity. Also, because of the potential temporal variability in the toxicity of effluents, a negative test result with a particular sample does not preclude the possibility that samples collected at some other time might exhibit acute (or chronic) toxicity.

2.6 The frequency with which acute toxicity tests are conducted under a given NPDES permit is determined by the regulatory agency on the basis of factors such as the variability and degree of toxicity of the waste, production schedules, and process changes.

2.7 Tests may be static (static non-renewal or static renewal), or flow-through.

2.7.1 STATIC TESTS

2.7.1.1 Static non-renewal tests - The test organisms are exposed to the same test solution for the duration of the test.

2.7.1.2 Static-renewal tests - The test organisms are exposed to a fresh solution of the same concentration of sample every 24 h or other prescribed interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers.

2.7.2 FLOW-THROUGH TESTS

2.7.2.1 Two types of flow-through tests are in common use: (1) sample is pumped continuously from the sampling point directly to the dilutor system; and (2) grab or composite samples are collected periodically, placed in a tank adjacent to the test laboratory, and pumped continuously from the tank to the dilutor system. The flow-through method employing continuous sampling is the preferred method for on-site tests. Because of the large volume (often 400 L/day) of effluent normally required for flow-through tests, it is generally considered too costly and impractical to conduct these tests off-site at a central laboratory.

2.8 Advantages and disadvantages of the types of tests are as follows:

2.8.1 STATIC NON-RENEWAL TESTS

2.8.1.1 Advantages:

1. Simple and inexpensive
2. Very cost effective in determining compliance with permit conditions.
3. Limited resources (space, manpower, equipment) required; would permit staff to perform many more tests in the same amount of time.
4. Smaller volume of effluent required than for static renewal or flow-through tests.

2.8.1.2 Disadvantages:

1. Dissolved oxygen (DO) depletion may result from high chemical oxygen demand (COD), biological oxygen demand (BOD), or metabolic wastes.
2. Possible loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Generally less sensitive than static renewal or flow-through tests, because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.8.2 STATIC-RENEWAL, ACUTE TOXICITY TESTS

2.8.2.1 Advantages:

1. Reduced possibility of dissolved oxygen (DO) depletion from high chemical oxygen demand (COD) and/or biological oxygen demand (BOD), or ill effects from metabolic wastes from organisms in the test solutions.
2. Reduced possibility of loss of toxicants through volatilization and/or adsorption to the exposure vessels.

3. Test organisms that rapidly deplete energy reserves are fed when the test solutions are renewed, and are maintained in a healthier state.

2.8.2.2 Disadvantages:

1. Require greater volume of effluent that non-renewal tests.
2. Generally less sensitive than flow-through tests, because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.8.3 FLOW-THROUGH TESTS

2.8.3.1 Advantages:

1. Provide a more representative evaluation of the acute toxicity of the source, especially if sample is pumped continuously directly from the source and its toxicity varies with time.
2. DO concentrations are more easily maintained in the test chambers.
3. A higher loading factor (biomass) may be used.
4. The possibility of loss of toxicant due to volatilization, adsorption, degradation, and uptake is reduced.

2.8.3.2 Disadvantages:

1. Large volumes of sample and dilution water are required.
2. Test equipment is more complex and expensive, and requires more maintenance and attention.
3. More space is required to conduct tests.
4. Because of the resources required, it would be very difficult to perform multiple or overlapping sequential tests.

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SECTION 3

HEALTH AND SAFETY

3.1 GENERAL PRECAUTIONS

3.1.1 Development and maintenance of an effective health and safety program in the laboratory requires an ongoing commitment by laboratory management, and includes (1) the appointment of a laboratory health and safety officer with the responsibility and authority to develop and maintain a safety program, (2) the preparation of a formal, written, health and safety plan, which is provided to each laboratory staff member, (3) an ongoing training program on laboratory safety, and (4) regularly scheduled, documented, safety inspections.

3.1.2 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to lack of oxygen or presence of noxious gases.

3.1.3 Prior to sample collection and laboratory work, personnel must determine that all required safety equipment and materials have been obtained and are in good condition.

3.1.4 Guidelines for the handling and disposal of hazardous materials must be strictly followed.

3.2 SAFETY EQUIPMENT

3.2.1 PERSONAL SAFETY GEAR

3.2.1.1 Personnel must use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes.

3.2.2 LABORATORY SAFETY EQUIPMENT

3.2.2.1 Each laboratory (including mobile laboratories) must be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, and eye fountains.

3.2.2.2 Mobile laboratories should be equipped with a telephone to enable personnel to summon help in case of emergency.

3.3 GENERAL LABORATORY AND FIELD OPERATIONS

3.3.1 Guidance in Material Safety Data Sheets should be followed for reagents and other chemicals purchased from supply houses. Incompatible materials should not be stored together.

3.3.2 Work with effluents must be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see Safety Manuals, Subsection 3.5). Personnel collecting samples and performing toxicity tests should not work alone.

3.3.3 Because the chemical composition of effluents is usually only poorly known, they must be considered as potential health hazards, and exposure to them should be minimized. Fume and canopy hoods over the test areas must be used whenever necessary.

3.3.4 It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

3.3.5 All containers must be adequately labeled to indicate their contents.

3.3.6 Strong acids and volatile organic solvents employed in glassware cleaning must be used in a fume hood or under an exhaust canopy over the work area.

3.3.7 Good housekeeping contributes to safety and reliable results.

3.3.8 Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.3.9 Mobile laboratories must be properly grounded to protect against electrical shock.

3.4 DISEASE PREVENTION

3.4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against hepatitis B, tetanus, typhoid fever, and polio.

3.5 SAFETY MANUALS

3.5.1 For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, check with the permittee and consult general industrial safety manuals, including USEPA (1986) and Walters and Jameson (1984).

3.6 WASTE DISPOSAL

3.6.1 Wastes generated during toxicity testing must be properly handled and disposed of in an appropriate manner. Each testing facility will have its own waste disposal requirements based on local, state, and Federal rules and regulations. It is extremely important that these rules and regulations be known, understood, and complied with by all persons responsible for, or otherwise involved in, performing testing activities. Local fire officials should be notified of any potentially hazardous conditions.

SECTION 4

QUALITY ASSURANCE

4.1 INTRODUCTION

4.1.1 Development and maintenance of a toxicity test laboratory quality assurance (QA) program requires an ongoing commitment by laboratory management, and includes the following: (1) appointment of a laboratory quality assurance officer with the responsibility and authority to develop and maintain a QA program; (2) preparation of a quality assurance plan with data quality objectives; (3) preparation of written descriptions of laboratory standard operating procedures (SOP's) for test organism culturing, toxicity testing, instrument calibration, sample chain-of-custody, laboratory sample tracking system, etc.; and (4) provision of adequate, qualified technical staff and suitable space and equipment to assure reliable data.

4.1.2 QA practices within an aquatic toxicology laboratory must address all activities that affect the quality of the final effluent toxicity data, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition and operation of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation.

4.1.3 Quality control practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance, and general guidance on good laboratory practices related to toxicity testing, see: FDA, 1978; USEPA, 1975; USEPA, 1979a; USEPA, 1980a; USEPA, 1980b; USEPA, 1991b; DeWoskin, 1984; and Taylor, 1987.

4.1.4 Guidance for the evaluation of laboratories performing toxicity tests and laboratory evaluation criteria may be found in USEPA, 1991b.

4.2 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

4.2.1 Separate test organism culturing and toxicity testing areas should be provided to avoid possible loss of cultures due to cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemical analysis laboratories or sample storage and preparation areas into organism culturing or toxicity testing areas, and from toxicity test laboratories and sample preparation areas into culture rooms.

4.2.2 Laboratory and toxicity test temperature control equipment must be adequate to maintain recommended test water temperatures. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent (see Section 5, Facilities and Equipment).

4.3 TEST ORGANISMS

4.3.1 The test organisms used in the procedures described in this manual are listed in Section 6, Test Organisms. The organisms should appear healthy,

behave normally, feed well, and have low mortality in cultures, during holding, and in test controls. Test organisms should be positively identified to species.

4.4 LABORATORY WATER USED FOR CULTURING AND TEST DILUTION WATER

4.4.1 The quality of water used for test organism culturing and for dilution water in toxicity tests is extremely important. Water for these two uses should come from the same source. The dilution water used in effluent toxicity tests will depend in part on the objectives of the study and logistical constraints, as discussed in detail in Section 7, Dilution Water. For tests performed to meet NPDES objectives, synthetic, moderately hard water should be used. The dilution water used for internal quality assurance tests with organisms, food, and reference toxicants should be the water routinely used with success in the laboratory. Types of water are discussed in Section 5, Facilities and Supplies. Water used for culturing and test dilution should be analyzed at least annually or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth for toxic metals and organics. The concentration of the metals, Al, As, Cr, Co, Cu, Fe, Pb, Ni, Zn, expressed as total metal, should not exceed 1 μg/L each, and Cd, Hg, and Ag, expressed as total metal, should not exceed 100 ng/L each. Total organochlorine pesticides plus PCBs should be less than 50 ng/L (APHA 1992). Individual pesticide concentrations should not exceed EPA's Ambient Water Quality chronic criteria values where available.

4.5 EFFLUENT SAMPLING AND SAMPLE HANDLING

4.5.1 Sample holding times and temperatures must conform to conditions described in Section 8, Effluent Sampling and Sample Handling.

4.6 TEST CONDITIONS

4.6.1 The temperature of test solutions must be measured by placing the thermometer or probe directly into the test solutions, or by placing the thermometer in equivalent volumes of water in surrogate vessels positioned at appropriate locations among the test vessels. Temperature should be recorded continuously in at least one vessel during the duration of each test. Test solution temperatures must be maintained within the limits specified for each test. DO concentration and pH in test chambers should be checked daily throughout the test period, as prescribed in Section 9, Acute Toxicity Test Procedures.

4.7 QUALITY OF TEST ORGANISMS

4.7.1 Where acute or short-term chronic toxicity tests are performed with effluents or receiving waters using test organisms obtained from outside the test laboratory, concurrent toxicity tests of the same type must be performed with a reference toxicant, unless the test organism supplier provides control chart data from at least the last five monthly acute toxicity tests using the same reference toxicity and test conditions.

4.7.2 The supplier should also certify the species identification of the test organisms, and provide the taxonomic reference (citation and page) or name(s) of the taxonomic expert(s) consulted.

4.7.3 If the laboratory performing toxicity tests maintains its own stock cultures, the sensitivity of the offspring should be determined in a toxicity test performed with a reference toxicant at least once each month (see Subsection 4.15). If preferred, this reference toxicant test may be performed concurrently with each effluent toxicity test. However, if a given species of test organism produced by inhouse cultures is used only monthly, or less frequently, in effluent toxicity tests, a reference toxicant test must be performed concurrently with the effluent toxicity test.

4.7.4 If a routine reference toxicant test fails to meet acceptability criteria, the test must be immediately repeated. If the failed reference toxicant test was being performed concurrently with an effluent or receiving water toxicity test, both tests must be repeated (for exception, see Subsection 4.16.5).

4.8 FOOD QUALITY

4.8.1 The nutritional quality of the food used in culturing and testing fish and invertebrates is an important factor in the quality of the toxicity test data. This is especially true for the unsaturated fatty acid content of brine shrimp nauplii, *Artemia*. Suitable trout chow, *Artemia*, and other foods must be obtained as described in this manual.

4.8.2 Problems with the nutritional suitability of the food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. If a batch of food is suspected to be defective, the performance of organisms fed with the new food can be compared with the performance of organisms fed with a food of known quality in side-by-side tests. If the food is used for culturing, its suitability should be determined using a short-term chronic test which will determine the effect of food quality on growth or reproduction of each of the relevant test species in culture, using four replicates with each food source. Where applicable, foods used only in acute toxicity tests can be compared with a food of known quality in side-by-side, multi-concentration acute tests, using the reference toxicant regularly employed in the laboratory QA program.

4.8.3 New batches of food used in culturing and testing should also be analyzed for toxic organics and metals or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. If the concentration of total organic chlorine exceeds 0.15 μg/g wet weight, or the total concentration of organochlorine pesticides plus PCBs exceeds 0.30 μg/g wet weight, or toxic metals exceed 20 μg/g wet weight, the food should not be used (for analytical methods see AOAC, 1990; USDA, 1989).

4.9 ACCEPTABILITY OF ACUTE TOXICITY TEST RESULTS

4.9.1 For the test results to be acceptable, control survival must equal or exceed 90%.

4.9.2 An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test condition summaries). The acceptability of the test will depend on the experience and professional judgment of the laboratory analyst and the reviewing staff of the regulatory authority. Any deviation from test specifications must be noted when reporting data from a test.

4.10 ANALYTICAL METHODS

4.10.1 Routine chemical and physical analyses for culture and dilution water, food, and test solutions must include established quality assurance practices outlined in Agency methods manuals (USEPA, 1979a; USEPA, 1993c).

4.10.2 Reagent containers should be dated when received from the supplier, and the shelf life should not be exceeded. Also, working solutions should be dated when prepared, and the recommended shelf life should be observed.

4.11 CALIBRATION AND STANDARDIZATION

4.11.1 Instruments used for routine measurements of chemical and physical parameters such as pH, DO, temperature, conductivity, salinity, alkalinity, and hardness must be calibrated and standardized prior to use each day according to the instrument manufacturer's procedures as indicated in the general section on quality assurance (see EPA Methods 150.1, 360.1, 170.1, and 120.1; USEPA, 1979b). Calibration data are recorded in a permanent log.

4.11.2 Wet chemical methods used to measure hardness, alkalinity, and total residual chlorine must be standardized prior to use each day according to the procedures for those specific EPA methods (see EPA Methods 130.2 and 310.1; USEPA 1979b).

4.12 REPLICATION AND TEST SENSITIVITY

4.12.1 The sensitivity of toxicity tests will depend in part on the number of replicates per concentration, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data.

4.13 VARIABILITY IN TOXICITY TEST RESULTS

4.13.1 Factors which can affect test success and precision include: the experience and skill of the laboratory analyst; test organism age, condition, and sensitivity; dilution water quality; temperature control; and the quality and quantity of food provided. The results will depend upon the species used and the strain or source of the test organisms, and test conditions such as temperature, DO, food, and water quality. The repeatability or precision of toxicity tests is also a function of the number of test organisms used at each toxicant concentration. Jensen (1972) discussed the relationship between

sample size (numbers of fish) and the standard error of the test, and considered 20 fish per concentration as optimum for Probit Analysis.

4.13.2 Test precision can be estimated by using the same strain of organisms under the same test conditions, and employing a known toxicant, such as a reference toxicant. The single-laboratory (intra-laboratory) and multi-laboratory (inter-laboratory) precision of acute toxicity tests with several common test species and reference toxicants are listed in Tables 1-4. Intra- and inter-laboratory precision are described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation, or CV) of the calculated endpoints from the replicated tests.

4.13.3 Intra-laboratory precision data from 268 acute toxicity tests with four species and five reference toxicants are listed in Tables 1 and 2. The precision, expressed as CV%, ranged from 3% to 86%. More recent CV values reported by Jop et al. (1986), Dorn and Rogers (1989), Hall et al. (1989), and Cowgill et al. (1990), fell in a somewhat lower range (8% to 41%).

4.13.4 Inter-laboratory precision of acute toxicity tests from 253 reference toxicant tests with seven species, listed in Tables 2, 3, 4, and 5 (expressed as CV%), ranged from 11% to 167%.

4.13.5 No clear pattern of differences were noted in the intra- or inter-laboratory test precision with the species listed, although the test results with some toxicants, such as cadmium, appear to be more variable than those with other reference toxicants.

4.13.6 Additional information on toxicity test precision is provided in the Technical Support Document for Water Quality-Based Toxics Control (see pp. 2-4, and 11-15; USEPA, 1991c).

4.14 DEMONSTRATING ACCEPTABLE LABORATORY PERFORMANCE

4.14.1 It is a laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with reference toxicants before it performs toxicity tests with effluents for permit compliance purposes. To meet this requirement, the intra-laboratory precision, expressed as percent coefficient of variation (CV%), of each type of test to be used in a laboratory should be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (i.e., the same test duration, type of dilution water, age of test organisms, feeding, etc.), and the same data analysis methods. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations.

4.15 DOCUMENTING ONGOING LABORATORY PERFORMANCE

4.15.1 Satisfactory laboratory performance is demonstrated by performing at least one acceptable test per month with a reference toxicant for each toxicity test method commonly used in the laboratory. For a given test method, successive tests must be performed with the same reference toxicant,

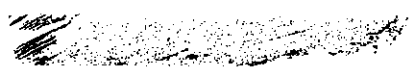


TABLE 1. INTRA-LABORATORY PRECISION OF LC50S FROM STATIC ACUTE TOXICITY TESTS WITH AQUATIC ORGANISMS USING REFERENCE TOXICANTS[1]

TEST ORGANISM	REFERENCE TOXICANT[2]								
	SDS			NAPCP			CD		
	N	LC50	CV(%)	N	LC50	CV(%)	N	LC50	CV(%)
Pimephales promelas (96 h, 21°C)[3]	9	8.6	20	12	0.14	40	9	0.15	120
Daphnia magna (24 h, 20°C)[4]	8	20.9	28	10	0.69	14	11	0.121	49
Daphnia magna (24 h, 26°C)[4]	10	12.9	48	9	0.67	25	9	0.026	77
Daphnia magna (48 h, 20°C)[4]	10	13.5	29	10	0.42	21	9	0.038	58
Daphnia magna (48 h, 26°C)[4]	9	10.8	33	9	0.48	23	8	0.009	35
Daphnia pulex (24 h, 20°C)[4]	9	18.4	23	9	0.64	15	5	0.147	30
Daphnia pulex (24 h, 26°C)[4]	10	13.9	25	9	0.62	25	10	0.063	45
Daphnia pulex (48 h, 20°C)[4]	10	12.6	32	9	0.48	16	10	0.042	45
Daphnia pulex (48 h, 26°C)[4]	9	10.2	36	8	0.47	32	6	0.006	14
Mysidopsis bahia (96 h, 25°C)[5]							13	0.346	9

[1]Precision expressed as percent coefficient of variation, where CV% = (standard deviation X 100)/mean.

[2]SDS = Sodium dodecyl (lauryl) sulfate; NAPCP = Sodium pentachlorophenate; CD = Cadmium; N = Number of tests; toxicant concentration in mg/L.

[3]Pimephales promelas tests were performed in soft, synthetic freshwater; total hardness, 40-48 mg/L as $CaCO_3$, by J. Dryer, Aquatic Biology Section, EMSL-Cincinnati.

[4]Daphnia data from Lewis and Horning, 1991. Tests with D. magna used hard reconstituted water (total hardness, 180-200 mg/L as $CaCO_3$); tests with D. pulex used moderately-hard reconstituted water (total hardness, 80-100 mg/L as $CaCO_3$).

[5]Mysid tests were performed in 25 ppt salinity, natural seawater. Data were provided by Steve Ward, Environmental Services Division, U.S. Environmental Protection Agency, Edison, New Jersey. Personal communication, November 14, 1990.

TABLE 2. INTRA- AND INTER-LABORATORY PRECISION OF ACUTE TOXICITY TESTS WITH *DAPHNIA MAGNA*, USING A STANDARD EFFLUENT[1,2]

LABORATORY	INTER-LABORATORY PRECISION: LC50s FROM REPLICATE TESTS		INTRA-LABORATORY PRECISION[3]
	24 H	48 H	
INDUSTRY			
1	14.4	4.2	
	11.4	4.9	---
2	13.9	6.8	
	16.6	6.1	
	13.7	6.1	6.4
3	11.7	3.5	
	17.4	7.1	---
GOVERNMENT			
1	14.0	4.4	
	10.0	4.4	
	10.8	4.1	4.0
2	13.2	4.5	
	14.1	4.5	---
3	11.6	4.2	---
COMMERCIAL			
1	20.1	4.9	
	20.1	4.7	---
2	8.9	3.7	
	12.3	5.6	---
3	14.8	9.0	
	25.4	9.1	
	26.4	8.6	3.0
N	20	20	3
MEAN	15.0	5.52	4.47
SD	4.75	1.75	1.75
CV%	31.6	31.6	39.1

[1]From Table 2, p. 191, Grothe and Kimerle, 1985. Tests performed at 20°C ± 2°C; dilution water hardness, 100 mg/L as $CaCO_3$; dilution water alkalinity, 76 mg/L as $CaCO_3$; effluent hardness, approx. 1000 mg/L as $CaCO_3$; effluent alkalinity, 310 mg/L as $CaCO_3$; effluent dilutions - 56%, 32%, 18%, 10%, 5.6%, 3.1%, 1.7%.

[2]LC50 expressed in percent effluent.

[3]Intra-laboratory precision expressed as the weighted mean CV(%).

TABLE 3. INTER-LABORATORY PRECISION OF ACUTE TOXICITY TESTS WITH AQUATIC ORGANISMS, USING REFERENCE TOXICANTS[1]

TEST ORGANISM	REFERENCE TOXICANT: SILVER N	SILVER LC50	SILVER CV(%)[2]	ENDOSULFAN N	ENDOSULFAN LC50	ENDOSULFAN CV(%)
1. Pimephales promelas (96 h, 22°C)						
96-h static test (Meas)	10	14.0	53	12	2.03	38
96-h flow-through test (Meas)	9	7.49	40	12	0.96	46
2. Oncorhyncus mykiss (96 h, 12°C)						
96-h static test (Meas)	10	34.5	88	12	1.15	50
96-h flow-through test (Meas)	9	11.5	33	12	0.40	42
3. Daphnia magna (48 h, 20°C)						
48-h static (Meas)	12	10.6	166	11	328	51
4. Mysidopsis bahia (96 h, 22°C)						
96-h static test (Nom)	6	210	27	5	0.84	62
96-h flow-through test (Nom)	6	251	22	6	1.02	58
96-h flow-through test (Meas)	6	192	58	5	0.94	167
5. Cyprinodon variegatus (96 h, 22°C)						
96-h static test (Nom)	4	1122	35	6	2.41	37
96-h flow-through test (Nom)	5	1573	50	6	1.69	46
96-h flow-through test (Meas)	5	1216	50	6	0.81	46

[1]Data for Pimephales promelas (fathead minnow), Oncorhyncus mykiss (rainbow trout), and Daphnia magna were taken from USEPA, 1983b.

Data for, Mysidopsis bahia, and Cyprinodon variegatus (sheepshead minnow) were taken from USEPA, 1981. Six laboratories participated in each study. Test salinity was 28‰.

LC50s expressed in µg/L.

In the studies with the freshwater organisms, the water hardness for five of the six laboratories ranged between 36 and 75 mg/L. However, the water hardness for the sixth laboratory was 255 mg/L, resulting in LC50 values for silver more than an order of magnitude larger than for the other five. These values were rejected in calculating the CV%. The mean weights of test fish were from 0.05-0.26 g for fathead minnows, and 0.22-1.32 g for rainbow trout. Daphnia were ≤ 24-h old.

In studies with the marine organisms, only one LC50 (presumably the combined LC50 from duplicate tests) was reported for each toxicity test. LC50s for flow-through tests with Mysidopsis bahia and Cyprinodon variegatus were calculated two different ways -- (1) on the basis of the nominal toxicant concentrations (Nom), and (2) on the basis of measured (Meas) toxicant concentrations. Test organism age was ≤ 2 days for Mysidopsis bahia, and 28 days for Cyprinodon variegatus. The salinity of test solutions was 28‰.

N, the total number of LC50 values used in calculating the CV(%) varied with organism and toxicant because some data were rejected due to water hardness, lack of concentration measurements, and/or because some of the LC50s were not calculable.

[2]CV% = Percent coefficient of variation = (standard deviation x 100)/mean.

TABLE 4. INTER-LABORATORY STUDY OF ACUTE TOXICITY TEST PRECISION, 1990: SUMMARY OF RESPONSES USING KCL AS THE REFERENCE TOXICANT[1]

TEST TYPE	NO. LABS SUBMITTING VALID DATA	TEST PRECISION (CV%)[2] GRAPH[3] METHOD N	LC50	CV%	STAT[4] METHOD N	LC50	CV%	TOTAL[5] N	LC50	CV%
Pimephales promelas (96 h, 22°C)[6]	17	6	944	28.8	13	832	27.8	17	864	29.6
Pimephales promelas (24 h, 25°C)[7]	6	6	832	11.5	6	832	11.5	-	-	-
Ceriodaphnia dubia (48 h, 25°C)[7]	11	11	256	53.1	11	264	48.5	-	-	-
Mysidopsis bahia (96 h, 22°C)[8]	14	7	292	32.9	11	250	36.0	14	268	37.3

[1] Interlaboratory study of toxicity test precision conducted in 1990 by the Environmental Monitoring Systems Laboratory-Cincinnati, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268, in cooperation with the states of New Jersey and North Carolina, and the Office of Water Enforcement and Permits, U.S. Environmental Protection Agency, Washington, DC.

[2] CV% Percent coefficient of variation = (standard deviation X 100)/mean. Calculated for LC50 from acute tests. LC50s expressed as mg/L KCl added to the dilution water.

[3] LC50 estimated by the Graphical Method.

[4] LC50 estimated by Probit, Litchfield-Wilcoxon, or Trimmed Spearman-Karber method.

[5] LC50 usually reported for only one method of analysis for each test. Where more than one LC50 was reported for a test, the lowest value was used to calculate the statistics for "Total."

[6] Data from the New Jersey Department of Environmental Protection: static daily-renewal tests, using moderately-hard synthetic freshwater.

[7] Data from North Carolina certified laboratories: static non-renewal tests, using moderately hard reconstituted freshwater.

[8] Data from the New Jersey Department of Environmental Protection: static daily-renewal tests, using 25 ppt salinity, FORTY FATHOMS® synthetic seawater.

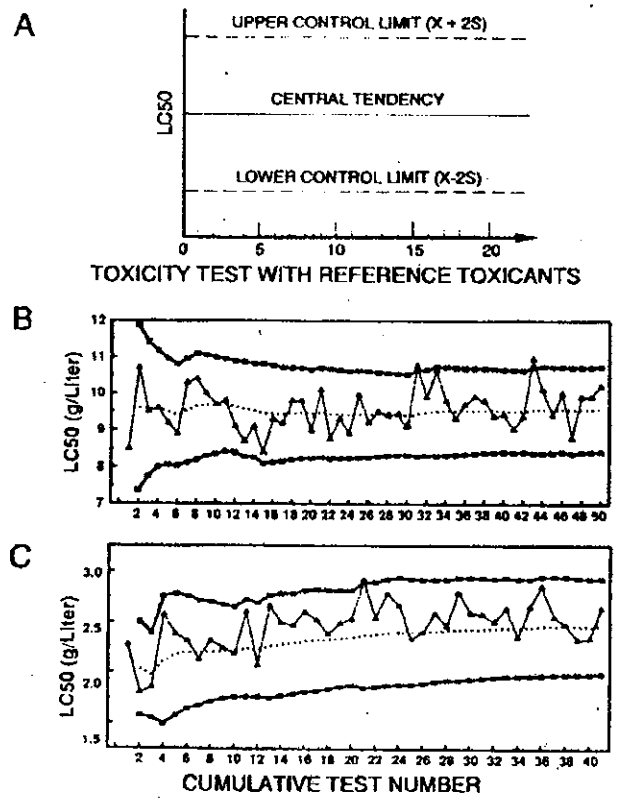


Figure 1. Control (cusum) charts: A, General case; B and C, 48-h acute tests with sodium chloride. (B) Fathead minnow (*Pimephales promelas*), and (C) *Ceriodaphnia dubia*, with the individual LC50s (Triangles), cumulative LC50 means (dotted line), and upper and lower control limits of two standard deviations (squares). (Provided by the Environmental Services Division, U.S. Environmental Protection Agency, Kansas City, KS).

toxicants for future release. Interested parties can determine the availability of "EPA Certified" reference toxicants by checking the EPA-Cincinnati electronic bulletin board, using a modem to access the following telephone number: 513-569-7610. Standard reference materials also can be obtained from commercial supply houses, or can be prepared inhouse using reagent grade chemicals. The regulatory agency should be consulted before reference toxicant(s) are selected and used.

4.17 RECORD KEEPING

4.17.1 Proper record keeping is important. A complete file should be maintained for each individual toxicity test or group of tests on closely related samples. This file should contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the toxicity test(s); chemical analysis data on the sample(s); detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions employed; and results of reference toxicant tests. Laboratory data should be recorded on a real-time basis to prevent the loss of information or inadvertent introduction of errors into the record. Original data sheets should be signed and dated by the laboratory personnel performing the tests.

4.17.2 The regulatory authority should retain records pertaining to discharge permits. Permittees are required to retain records pertaining to permit applications and compliance for a minimum of 3 years [40 CFR 122.41(j)(2)].

SECTION 5

FACILITIES AND EQUIPMENT

5.1 GENERAL REQUIREMENTS

5.1.1 Effluent toxicity tests may be performed in a fixed or mobile laboratory. Facilities should include equipment for rearing and/or holding organisms.

5.1.2 The facilities must be well ventilated and free of toxic fumes. Sample preparation, culturing, and toxicity testing areas should be separated to avoid cross contamination of cultures or toxicity test solutions with toxic fumes. Laboratory ventilation systems should be checked to ensure that return air from chemistry laboratories and/or sample handling areas is not circulated to test organism culture rooms or toxicity test rooms, or that air from toxicity test rooms does not contaminate culture areas. Air pressure differentials between such rooms should not result in a net flow of potentially contaminated air to sensitive areas through open or loosely-fitting doors.

5.1.3 Control of test solution temperature can best be achieved using circulating water baths, heat exchangers, or environmental chambers. Photoperiod can be controlled using automatic timers in the laboratory or environmental chambers.

5.1.4 Water used for rearing, holding, and testing organisms may be reconstituted synthetic water, ground water, surface water, or dechlorinated tap water. Dechlorination can be accomplished by carbon filtration, laboratory water conditioning units, or the use of sodium thiosulfate. After dechlorination, total residual chlorine should be non-detectable. Sodium thiosulfate may be toxic to the test organisms, and if used for dechlorination, paired controls with and without sodium thiosulfate should be incorporated in effluent toxicity tests. Use of 3.6 mg (anhydrous) sodium thiosulfate/L will reduce 1.0 mg chlorine/L. After dechlorination, total residual chlorine should be non-detectable.

5.1.4.1 A deionizing system providing 18 mega-ohm, laboratory grade water should be provided with sufficient capacity for laboratory needs. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade water deionizer with preconditioned water from a CULLIGEN®, CONTINENTAL®, or equivalent, mixed-bed water treatment system.

5.1.5 Air used for aeration must be free of oil and fumes. Oil-free air pumps should be used where possible. Particulates can be removed from the air using BALSTON® Grade BX or equivalent filters (Balston, Inc., Lexington, MA), and oil and other organic vapors can be removed using activated carbon filters (BALSTON®, C-1 filter, or equivalent).

5.1.6 During rearing, holding, and testing, test organisms should be shielded from external disturbances such as rapidly changing light conditions (especially salmonids) and pedestrian traffic.

5.1.7 Materials used for exposure chambers, tubing, etc., that come in contact with the effluent and dilution water should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON®) should be used whenever possible to minimize sorption and leaching of toxic substances, and may be reused after cleaning. Containers made of plastics, such as polyethylene, polypropylene, polyvinyl chloride, TYGON®, etc., may be used to ship, store, and transfer effluents and receiving waters, but they should not be reused unless absolutely necessary, because they could carry over adsorbed toxicants from one test to another. However, these containers may be repeatedly reused for storing uncontaminated waters such as deionized or laboratory-prepared dilution waters and receiving waters. Glass or disposable polystyrene containers can be used as test chambers. The use of large (≥ 20 L) glass carboys is discouraged for safety reasons.

5.1.8 New plastic products should be tested for toxicity before general use by exposing organisms to them under ordinary test conditions.

5.1.9 Equipment which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below. Fiberglass, in addition to the previously mentioned materials, can be used for holding and dilution water storage tanks, and in the water delivery system. All material should be flushed or rinsed thoroughly with dilution water before using in the test.

5.1.10 Copper, galvanized material, rubber, brass, and lead must not come in contact with holding or dilution water, or with effluent samples and test solutions. Some materials, such as neoprene rubber (commonly used for stoppers), may be toxic and should be tested before use.

5.1.11 Silicone adhesive used to construct glass test chambers absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

5.2 CLEANING TEST CHAMBERS AND LABORATORY APPARATUS

5.2.1 New plasticware used for effluent or dilution water collection or organism test chambers does not require thorough cleaning before use. It is sufficient to rinse new sample containers once with sample dilution water before use. New glassware must be soaked overnight in 10% acid (see below) and rinsed well in deionized water and dilution water.

5.2.2 All non-disposable sample containers, test vessels, tanks, and other equipment that has come in contact with effluent must be washed after use in the manner described below to remove surface contaminants as described below:

1. Soak 15 min in tap water, and scrub with detergent, or clean in an automatic dishwasher.

2. Rinse twice with tap water.
3. Carefully rinse once with fresh, dilute (10%, V:V) hydrochloric or nitric acid to remove scale, metals, and bases. To prepare a 10% solution of acid, add 10 mL of concentrated acid to 90 mL of deionized water.
4. Rinse twice with deionized water.
5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).
6. Rinse three times with deionized water.

5.2.3 All test chambers and equipment should be thoroughly rinsed with the dilution water immediately prior to use in each test.

5.3 APPARATUS AND EQUIPMENT FOR CULTURING AND TOXICITY TESTS

5.3.1 Culture units -- see Appendix. It is preferable to obtain test organisms from in-house culture units. If it is not feasible to maintain cultures in-house, test organisms can be obtained from commercial sources, and should be shipped to the laboratory in well oxygenated water in insulated containers to minimize excursions in water temperature during shipment. The temperature of the water in the shipping containers should be measured on arrival, to determine if the organisms were subjected to obvious undue thermal stress.

5.3.2 Samplers -- automatic samplers, preferably with sample cooling capability, that can collect a 24-h composite sample of 2 L or more.

5.3.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

5.3.4 Environmental chamber or equivalent facility with temperature control (20°C or 25°C)

5.3.5 Water purification system -- MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$, or equivalent. Depending on the quantity of high grade water needed, a first-stage pre-conditioner deionizer, such as a CULLIGEN® or CONTINENTAL® System, or equivalent, may be needed to provide feed water to the high-purity system.

5.3.6 Balance -- analytical, capable of accurately weighing to 0.0001 g.

5.3.7 Reference weights, Class S -- for documenting the performance of the analytical balance(s). The balance(s) should be checked with reference weights which are at the upper and lower ends of the range of the weighings made when the balance is used. A balance should be checked at the beginning of each series of weighings, periodically (such as every tenth weight) during a long series of weighings, and after the last weight of a series is taken.

5.3.8 Test chambers -- borosilicate glass or non-toxic disposable plastic test chambers are suitable. Test chamber volumes are indicated in the method summaries. To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic, 6 mm (1/4 in) thick.

5.3.9 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

5.3.10 Volumetric pipets -- Class A, 1-100 mL.

5.3.11 Serological pipets -- 1-10 mL, graduated.

5.3.12 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

5.3.13 Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring test organisms.

5.3.14 Wash bottles -- for rinsing small glassware and instrument electrodes and probes.

5.3.15 Glass or electronic thermometers -- for measuring water temperature.

5.3.16 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

5.3.17 National Bureau of Standards Certified thermometer (see USEPA Method 170.1; USEPA 1979b).

5.3.18 pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

5.3.19 Refractometer -- for measuring effluent, receiving, and test solution salinity.

5.3.20 Amperometric titrator -- for measuring total residual chlorine.

5.4 REAGENTS AND CONSUMABLE MATERIALS

5.4.1 Reagent water -- defined as MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$ or equivalent water (see Subsection 5.3.5 above).

5.4.2 Effluent, dilution water, and receiving water -- see Section 7, Dilution Water, and Section 8, Effluent and Receiving Water Sampling and Sample Handling.

5.4.3 Reagents for hardness and alkalinity tests (see USEPA Methods 130.2 and 310.1; USEPA 1979b).

5.4.4 Standard pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for instrument calibration (see USEPA Method 150.1; USEPA 1979b).

5.4.5 Specific conductivity and salinity standards (see USEPA Method 120.1; USEPA 1979b).

5.4.6 Laboratory quality control check samples and standards for the above chemistry methods.

5.4.7 Reference toxicant solutions (see Section 4, Quality Assurance).

5.4.8 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1; USEPA 1979b), or reagents for modified Winkler analysis.

5.4.9 Sources of Food for Cultures and Toxicity Tests.

5.4.9.1 All food should be tested for nutritional suitability, and chemically analyzed for organic chlorine, PCBs, and toxic metals (see Section 4, Quality Assurance).

5.4.9.2 Brine Shrimp (*Artemia*) -- see Appendix A.

1. Brine Shrimp (*Artemia*) Cysts.

   There are many commercial sources of brine shrimp cysts. Sources include: Aquarium Products, 180L Penrod Ct., Glen Burnie, MD 21061); San Francisco Bay Brand, 8239 Enterprise Dr., Newark, CA 94560 (Phone: 415-792-7200); Argent Aquaculture, 8702 152nd Ave, N.E., Redmond, WA 98052 (206-885-3777)(Argentina brine shrimp eggs, Grade 1, Gold Label); and Jungle, Inc. Additional sources are listed in the section on *Artemia* culture in Appendix A. The quality of the cysts may vary from one batch to another, and the cysts in each new batch (can or lot) should be evaluated for nutritional suitability and chemical contamination. The nutritional suitability (see Leger et al., 1985, 1986) of each new batch is checked against known suitable reference cysts by performing a side-by-side growth and/or reproduction tests using the "new" and "reference" cysts. If the results of tests for nutritional suitability or chemical contamination do not meet standards, the *Artemia* should not be used.

2. Frozen Adult Brine Shrimp

   Frozen adult brine shrimp are available from San Francisco Bay Brand, 8239 Enterprise Dr., Newark, CA 94560 (415-792-7200).

5.4.9.3 Trout Chow

Starter or No. 1 pellets, prepared according to current U.S. Fish and Wildlife Service specifications, are available from: Zeigler Bros., Inc., P.O. Box 95, Gardners, PA 17324 (717-780-9009); Glencoe Mills, 1011 Elliott, Glencoe, MN 55336 (612-864-3181); and Murray Elevators, 118 West 4800 South, Murray, UT 84107 (800-521-9092). (The flake food, TETRAMIN® or BIORIL®, can be used regularly as a substitute for trout chow in preparing food for daphnids, and can be used as a short-term substitute for trout chow in feeding fathead minnows.)

5.4.9.4 Dried, Powdered Leaves (CEROPHYLL®)

Dried, powdered, cereal leaves are available from Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 (800-325-3010); or as CEROPHYLL®, from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692-9012 (716-359-2502). Dried, powdered, alfalfa leaves obtained from health food stores have been found to be a satisfactory substitute for cereal leaves.

5.4.9.5 Yeast

Packaged dry yeast, such as FLEISCHMANN'S®, or equivalent, can be purchased at the local grocery store or is available from Lake States Yeast, Rhineland, WI.

5.4.9.6 Flake Fish Food

The flake foods, TETRAMIN® and BIORIL®, are available at most pet supply shops.

5.5 **TEST ORGANISMS**

5.5.1 Test organisms are obtained from inhouse cultures or commercial suppliers (see Section 6, Test Organisms).

SECTION 6

TEST ORGANISMS

6.1 TEST SPECIES

6.1.1 The species used in characterizing the acute toxicity of effluents and/or receiving waters will depend on the requirements of the regulatory authority and the objectives of the test. It is essential that good quality test organisms be readily available throughout the year from inhouse or commercial sources to meet NPDES monitoring requirements. The organisms used in toxicity tests must be identified to species. If there is any doubt as to the identity of the test organisms, representative specimens should be sent to a taxonomic expert to confirm the identification.

6.1.2 Toxicity test conditions and culture methods are provided in this manual for the following principal test organisms:

Freshwater Organisms:

1. *Ceriodaphnia dubia* (daphnid) (Table 11).
2. *Daphnia pulex* and *D. magna* (daphnids) (Table 12).
3. *Pimephales promelas* (fathead minnow) (Table 13).
4. *Oncorhynchus mykiss* (rainbow trout) and *Salvelinus fontinalis* (brook trout) (Table 14).

Estuarine and Marine Organisms:

1. *Mysidopsis bahia* (mysid) (Table 15).
2. *Cyprinodon variegatus* (sheepshead minnow) (Table 16).
3. *Menidia beryllina* (inland silverside), *M. menidia* (Atlantic silverside), and *M. peninsulae* (tidewater silverside) (Table 17).

6.1.3 The test species listed in Subsection 6.1.2 are the recommended acute toxicity test organisms. They are easily cultured in the laboratory, are sensitive to a variety of pollutants, and are generally available throughout the year from commercial sources. Summaries of test conditions for these species are provided in Tables 11-17. Guidelines for culturing and/or holding the organisms are provided in Appendix A.

6.1.4 Additional species may be suitable for toxicity tests in the NPDES Program. A list of alternative acute toxicity test species and minimal testing requirements (i.e., temperature, salinity, and life stage) for these species are provided in Appendix B. It is important to note that these species may not be as easily cultured or tested as the species on the list in Subsection 6.1.2, and may not be available from commercial sources.

6.1.5 Some states have developed culturing and testing methods for indigenous species that may be as sensitive or more sensitive than the species recommended in Subsection 6.1.2. However, EPA allows the use of indigenous species only where state regulations require their use or prohibit importation

of the species in Subsection 6.1.2. Where state regulations prohibit importation or use of the recommended test species, permission must be requested from the appropriate state agency prior to their use.

6.1.6 Where states have developed culturing and testing methods for indigenous species other than those recommended in this manual, data comparing the sensitivity of the substitute species and one or more of the recommended species must be obtained in side-by-side toxicity tests with reference toxicants and/or effluents, to ensure that the species selected are at least as sensitive as the recommended species. These data must be submitted to the permitting authority (State or Region) if required. EPA acknowledges that reference toxicants prepared from pure chemicals may not always be representative of effluents. However, because of the observed and/or potential variability in the quality and toxicity of effluents, it is not possible to specify a representative effluent.

6.1.7 Guidance for the selection of test organisms where the salinity of the effluent and/or receiving water requires special consideration is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991d).

1. Where the salinity of the receiving water is <1‰, freshwater organisms are used regardless of the salinity of the effluent.
2. Where the salinity of the receiving water is ≥1‰, the choice of organisms depends on state water quality standards and/or permit requirements.

6.2 SOURCES OF TEST ORGANISMS

6.2.1 INHOUSE CULTURES

6.2.1.1 Inhouse cultures should be established wherever it is cost effective. If inhouse cultures cannot be maintained, test organisms should be purchased from experienced commercial suppliers (see Appendix for sources).

6.2.2 COMMERCIAL SUPPLIERS

6.2.2.1 All of the principal test organisms listed in Subsection 6.1.2 are available from commercial suppliers.

6.2.3 FERAL (NATURAL OCCURRING, WILD CAUGHT) ORGANISMS

6.2.3.1 The use of test organisms taken from the receiving water has strong appeal, and would seem to be the logical approach. However, it is impractical for the following reasons:

1. Sensitive organisms may not be present in the receiving water because of previous exposure to the effluent or other pollutants.
2. It is often difficult to collect organisms of the required age and quality from the receiving water;
3. Most states require collection permits, which may be difficult to obtain. Therefore, it is usually more cost effective to culture the

organisms in the laboratory or obtain them from private, state, or Federal sources. Fish such as fathead minnows, sheepshead minnows, and silversides, and invertebrates such as daphnids and mysids, are easily reared in the laboratory or purchased.

4. The required QA/QC records, such as the single laboratory precision data, would not be available.
5. Since it is mandatory that the identity of test organisms is known to the species level, it would necessary to examine each organism caught in the wild to confirm its identity, which would usually be impractical or, at the least, very stressful to the organisms.
6. Test organisms obtained from the wild must be observed in the laboratory for a minimum of one week prior to use, to assure that they are free of signs of parasitic or bacterial infections and other adverse effects. Fish captured by electroshocking must not be used in toxicity testing.

6.2.3.2 Guidelines for collection of feral organisms are provided in USEPA, 1973; USEPA 1990a.

6.2.4 Regardless of their source, test organisms should be carefully observed to ensure that they are free of signs of stress and disease, and in good physical condition. Some species of test organisms, such as trout, can be obtained from stocks certified as "disease-free."

6.3 LIFE STAGE

6.3.1 Young organisms are often more sensitive to toxicants than are adults. For this reason, the use of early life stages, such as first instars of daphnids and juvenile mysids and fish, is recommended for all tests. There may be special cases, however, where the limited availability of organisms will require some deviation from the recommended life stage. In a given test, all organisms should be approximately the same age and should be taken from the same source. Since age may affect the results of the tests, it would enhance the value and comparability of the data if the same species in the same life stages were used throughout a monitoring program at a given facility.

6.4 LABORATORY CULTURING

6.4.1 Instructions for culturing and/or holding the recommended test organisms are included in Appendix A.

6.5 HOLDING AND HANDLING TEST ORGANISMS

6.5.1 Test organisms should not be subjected to changes of more than 3°C in water temperature or 3‰ in salinity in any 12 h period.

6.5.2 Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress. Organisms that are dropped or touch dry surfaces or are injured during handling must be discarded. Dipnets are best for handling larger organisms. These nets are commercially available or can be made from

small-mesh nylon netting, silk bolting cloth, plankton netting, or similar material. Wide-bore, smooth glass tubes (4 to 8 mm inside diameter) with rubber bulbs or pipettors (such as a PROPIPETTE® or other pipettor) should be used for transferring smaller organisms such as daphnids, mysids, and larval fish.

6.5.3 Holding tanks for fish are supplied with a good quality water (see Section 5, Facilities and Equipment) with a flow-through rate of at least two tank-volumes per day. Otherwise, use a recirculation system where the water flows through an activated carbon or undergravel filter to remove dissolved metabolites. Culture water can also be piped through high intensity ultraviolet light sources for disinfection, and to photodegrade dissolved organics.

6.5.4 Crowding should be avoided. The DO must be maintained at a minimum of 4.0 mg/L for marine and warm water, freshwater species, and 6.0 mg/L for cold-water, freshwater species. The solubility of oxygen depends on temperature, salinity, and altitude. Aerate if necessary.

6.5.5 Fish should be fed as much as they will eat at least once a day with live or frozen brine shrimp or dry food (frozen food should be completely thawed before use). Brine shrimp can be supplemented with commercially prepared food such as TETRAMIN® or BIORIL® flake food, or equivalent. Excess food and fecal material should be removed from the bottom of the tanks at least twice a week by siphoning.

6.5.6 Fish should be observed carefully each day for signs of disease, stress, physical damage, and mortality. Dead and abnormal specimens should be removed as soon as observed. It is not uncommon to have some fish (5-10%) mortality during the first 48 h in a holding tank because of individuals that refuse to feed on artificial food and die of starvation.

6.5.7 A daily record of feeding, behavioral observations, and mortality should be maintained.

6.6 **TRANSPORTATION TO THE TEST SITE**

6.6.1 Organisms are transported from the base or supply laboratory to a remote test site in culture water or standard dilution water in plastic bags or large-mouth screw-cap (500 mL) plastic bottles in styrofoam coolers. Adequate DO is maintained by replacing the air above the water in the bags with oxygen from a compressed gas cylinder, and sealing the bags. Another method commonly used to maintain sufficient DO during shipment is to aerate with an airstone which is supplied from a portable pump. The DO concentration must not fall below 4.0 mg/L for marine and warm-water, freshwater species, and 6.0 mg/L for cold-water, freshwater species.

6.6.2 Upon arrival at the test site, organisms are transferred to receiving water if receiving water is to be used as the test dilution water. All but a small volume of the holding water (approximately 5%) is removed by siphoning, and replaced slowly over a 10 to 15 min period with dilution water. If receiving water is used as dilution water, caution must be exercised in

exposing the test organisms to it, because of the possibility that it might be toxic. For this reason, it is recommended that only approximately 10% of the test organisms be exposed initially to the dilution water. If this group does not show excessive mortality or obvious signs of stress in a few hours, the remainder of the test organisms are transferred to the dilution water.

6.6.3 A group of organisms must not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed, or if mortality appears to exceed 10% preceding the test. If the organisms fail to meet these criteria, the entire group must be discarded and a new group obtained. The mortality may be due to the presence of toxicity, if receiving water is used as dilution water, rather than a diseased condition of the test organisms. If the acclimation process is repeated with a new group of test organisms and excessive mortality occurs, it is recommended that an alternative source of dilution water be used.

6.6.4 In static tests, marine organisms can be used at all concentrations of effluent by adjusting the salinity of the effluent to a standard salinity (such as 25‰) or to the salinity approximating that of the receiving water, by adding sufficient dry ocean salts, such as FORTY FATHOMS®, or equivalent, GP2 or hypersaline brine.

6.6.5 Saline dilution water can be prepared with deionized water or a freshwater such as well water or a suitable surface water. If dry ocean salts are used, care must be taken to ensure that the added salts are completely dissolved and the solution is aerated 24 h before the test organisms are placed in the solutions. The test organisms should be acclimated in synthetic saline water prepared with the dry salts. Caution: addition of dry ocean salts to dilution water may result in an increase in pH. (The pH of estuarine and coastal saline waters is normally 7.5-8.3.)

6.6.6 All effluent concentrations and the control(s) used in a test should have the same salinity. However, if this is impractical because of the large volumes of water required, such as in flow-through tests, the highest effluent concentration (lowest salinity) that could be tested would depend upon the salinity of the receiving water and the tolerance of the test organisms. The required salinities for toxicity tests with estuarine and marine species are listed in Tables 15-17. However, the tolerances of other candidate test species would have to be determined by the investigator in advance of the test.

6.6.7 Because of the circumstances described above, when performing flow-through tests of effluents discharged to saline waters, it is advisable to acclimate groups of test organisms to each of three different salinities, such as 10, 20, and 30‰, prior to transporting them to the test site. It may also be advisable to maintain cultures of these test organisms at a series of salinity levels, including at least 10, 20, and 30‰, so that the change in salinity upon acclimation at the desired test dilutions does not exceed 6‰.

6.7 TEST ORGANISM DISPOSAL

6.7.1 When the toxicity test is concluded, all test organisms (including controls) should be humanely destroyed and disposed of in an appropriate manner.

SECTION 7

DILUTION WATER

7.1 TYPES OF DILUTION WATER

7.1.1 The type of dilution water used in effluent toxicity tests will depend largely on the objectives of the study:

7.1.1.1. If the objective of the test is to estimate the absolute acute toxicity of the effluent, which is a primary objective of NPDES permit-related toxicity testing, a synthetic (standard) dilution water is used. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.2. If the objective of the test is to estimate the acute toxicity of the effluent in uncontaminated receiving water, the test may be conducted using dilution water consisting of a single grab sample of receiving water (if non-toxic), collected either upstream and outside the influence of the outfall, or with other uncontaminated natural water (ground or surface water) or standard dilution water having approximately the same characteristics (hardness and/or salinity) as the receiving water. Seasonal variations in the quality of surface waters may affect effluent toxicity. Therefore, the hardness of fresh receiving water, and the salinity of saline receiving water samples should be determined before each use. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.3. If the objective of the test is to determine the additive or mitigating effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected immediately upstream or outside the influence of the outfall. A second set of controls, using culture water, should be included in the test.

7.2 STANDARD, SYNTHETIC DILUTION WATER

7.2.1 Standard, synthetic dilution water is prepared with deionized water and reagent grade chemicals or mineral water (Tables 6 and 7) and commercial sea salts (FORTY FATHOMS®, HW MARINEMIX®) (Table 8). The source water for the deionizer can be groundwater or tap water.

7.2.2 DEIONIZED WATER USED TO PREPARE STANDARD, SYNTHETIC, DILUTION WATER

7.2.2.1 Deionized water is obtained from a MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$, or equivalent system. It is advisable to provide a preconditioned (deionized) feed water by using a CULLIGAN®, CONTINENTAL®, or equivalent, system in front of the MILLIPORE® System to extend the life of the MILLIPORE® cartridges.

7.2.2.2 The recommended order of the cartridges in a four-cartridge deionizer (i.e., MILLI-Q® System or equivalent) is: (1) ion exchange, (2) ion exchange,

(3) carbon, and (4) organic cleanup (such as ORGANEX-Q®, or equivalent), followed by a final bacteria filter. The QPAK™$_2$ water system is a sealed system which does not allow for the rearranging of the cartridges. However, the final cartridge is an ORGANEX-Q® filter, followed by a final bacteria filter. Commercial laboratories using this system have not experienced any difficulty in using the water for culturing or testing. Reference to the MILLI-Q® systems throughout the remainder of the manual includes all MILLIPORE® or equivalent systems.

7.2.3 STANDARD, SYNTHETIC FRESHWATER

7.2.3.1 To prepare 20 L of standard, synthetic, moderately hard, reconstituted water, use the reagent grade chemicals in Table 6 as follows:

1. Place 19 L of MILLI-Q®, or equivalent, deionized water in a properly cleaned plastic carboy.
2. Add 1.20 g of $MgSO_4$, 1.92 g $NaHCO_3$, and 0.080g KCl to the carboy.
3. Aerate overnight.
4. Add 1.20 g of $CaSO_4 \cdot 2 H_2O$ to 1 L of MILLI-Q® or equivalent deionized water in a separate flask. Stir on magnetic stirrer until calcium sulfate is dissolved, add to the 19 L above, and mix well.
5. For *Ceriodaphnia* culture and testing, add sufficient sodium selenate (Na_2SeO_4) to provide 2 μg selenium per liter of final dilution water.
6. Aerate the combined solution vigorously for an additional 24 h to dissolve the added chemicals and stabilize the medium.
7. The measured pH, hardness, etc., should be as listed in Table 6.

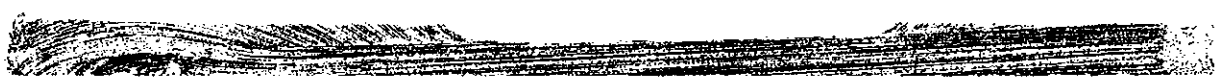
7.2.3.2 To prepare 20 L of standard, synthetic, moderately hard, reconstituted water, using 20% mineral water such as PERRIER® Water, or equivalent (Table 7), follow the instructions below.

1. Place 16 L of MILLI-Q® or equivalent deionized water in a properly cleaned plastic carboy.
2. Add 4 L of PERRIER® Water, or equivalent.
3. Aerate vigorously for 24 h to stabilize the medium.
4. The measured pH, hardness, and alkalinity of the aerated water will be as indicated in Table 7.
5. This synthetic water is referred to as diluted mineral water (DMW) in the toxicity test methods.

7.2.4 STANDARD, SYNTHETIC SEAWATER

7.2.4.1 To prepare 20 L of a standard, synthetic, reconstituted seawater (modified GP2), with a salinity of 31‰ (Table 8), follow the instructions below. Other salinities can be prepared by making the appropriate dilutions.

1. Place 20 L of MILLI-Q® or equivalent deionized water in a properly cleaned plastic carboy.
2. Weigh reagent grade salts listed in Table 8 and add, one at a time, to the deionized water. Stir well after adding each salt.
3. Aerate the final solution at a rate of 1 L/h for 24 h.
4. Check the pH and salinity.



Larger or smaller volumes of modified GP2 can be prepared by using proportionately larger or smaller amounts of salts and dilution water.

7.2.4.2 Synthetic seawater can also be prepared by adding commercial sea salts, such as FORTY FATHOMS®, HW MARINEMIX® or equivalent, to deionized water. For example, thirty-one parts per thousand (31‰) FORTY FATHOMS® can be prepared by dissolving 31 g of product per liter of deionized water. The salinity of the resulting solutions should be checked with a refractometer.

7.3 USE OF RECEIVING WATER AS DILUTION WATER

7.3.1 If the objectives of the test require the use of uncontaminated surface water as dilution water, and the receiving water is uncontaminated, it may be possible to collect a sample of the receiving water close to the outfall, but upstream from or beyond the influence of the effluent. However, if the receiving water is contaminated, it may be necessary to collect the sample in an area "remote" from the discharge site, matching as closely as possible the physical and chemical characteristics of the receiving water near the outfall.

7.3.2 The sample should be collected immediately prior to the test, but never more than 96 h before the test begins. Except where it is used within 24 h, or in the case where large volumes are required for flow-through tests, the sample should be chilled to 4°C during or immediately following collection, and maintained at that temperature prior to use in the test.

7.3.3 In the case of freshwaters, the regulatory authority may require that the hardness of the dilution water be comparable to the receiving water at the discharge site. This requirement can be satisfied by collecting an uncontaminated surface water with a suitable hardness, or adjusting the hardness of an otherwise suitable surface water by addition of reagents as indicated in Table 6.

7.3.4 In an estuarine environment, the investigator should collect uncontaminated water having a salinity as near as possible to the salinity of the receiving water at the discharge site. Water should be collected at slack high tide, or within one hour after high tide. If there is reason to suspect contamination of the water in the estuary, it is advisable to collect uncontaminated water from an adjacent estuary. At times it may be necessary to collect water at a location closer to the open sea, where the salinity is relatively high. In such cases, deionized water or uncontaminated freshwater is added to the saline water to dilute it to the required test salinity. Where necessary, the salinity of a surface water can be increased by the addition of artificial sea salts, such as FORTY FATHOMS® or equivalent, a natural seawater of higher salinity, or hypersaline brine. Instructions for the preparation of hypersaline brine by concentrating natural seawater are provided below.

7.3.5 Receiving water containing debris or indigenous organisms, that may be confused with or attack the test organisms, should be filtered through a sieve having 60 μm mesh openings prior to use.

TABLE 6. PREPARATION OF SYNTHETIC FRESHWATER USING REAGENT GRADE CHEMICALS[1]

Water Type	Reagent Added (mg/L)[2]				Final Water Quality		
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	pH[3]	Hardness[4]	Alkalinity[4]
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	60-70
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

[1]Taken in part from Marking and Dawson (1973).
[2]Add reagent grade chemicals to deionized water.
[3]Approximate equilibrium pH after 24 h of aeration.
[4]Expressed as mg $CaCO_3$/L.

TABLE 7. PREPARATION OF SYNTHETIC FRESHWATER USING MINERAL WATER[1]

Water Type	Volume of Mineral Water Added (mL/L)[2]	Proportion of Mineral Water (%)	Final Water Quality		
			pH[3]	Hardness[4]	Alkalinity[4]
Very soft	50	2.5	7.2-8.1	10-13	10-13
Soft	100	10.0	7.9-8.3	40-48	30-35
Moderately Hard	200	20.0	7.9-8.3	80-100	60-70
Hard	400	40.0	7.9-8.3	160-180	110-120
Very hard[5]	---	---	---	---	---

[1]From Mount et al., 1987; data provided by Philip Lewis, EMSL-Cincinnati.
[2]Add mineral water to MILLI-Q® water or equivalent to prepare DMW (Diluted Mineral Water).
[3]Approximate equilibrium pH after 24 h of aeration.
[4]Expressed as mg $CaCO_3$/L.
[5]Dilutions of PERRIER® Water form a precipitate when concentrations equivalent to "very hard water" are aerated.

TABLE 8. PREPARATION OF SYNTHETIC SEAWATER USING REAGENT GRADE CHEMICALS[1,2,3]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
KCl	0.61	12.2
KBr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$	0.17	3.40

[1]Modified GP2.

[2]The constituent salts and concentrations were taken from USEPA, 1990b. The salinity is 30.89 g/L.

[3]GP2 can be diluted with deionized (DI) water to the desired test salinity.

7.3.6 When receiving water is used as dilution water in flow-through tests, it is preferable to pump the dilution water continuously to the acclimation chamber and/or dilutor. However, where it is not feasible to pump the dilution water continuously, grab samples of the dilution water are transported to the test site in tanks, and continuously pumped from the tanks to the acclimation chamber and/or dilutor.

7.3.7 HYPERSALINE BRINE

7.3.7.1 Hypersaline brine (HSB) has several advantages that make it desirable for use in toxicity testing. It can be made from any high quality, filtered seawater by evaporation, and can be added to deionized water to prepare dilution water, or to effluents or surface waters to increase their salinity.

7.3.7.2 The ideal container for making HSB from natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method used is a thermostatically controlled heat exchanger made from fiberglass. If aeration is used, use only oil-free air compressors to prevent contamination.

7.3.7.3 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several thorough deionized water rinses. High quality (and preferably high salinity) seawater should be filtered to at least 10 μm before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

7.3.7.4 The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

7.3.7.5 After the required salinity is attained, the HSB should be filtered a second time through a 1-μm filter and poured directly into portable containers (20-L CUBITAINERS® or polycarbonate water cooler jugs are suitable). The containers should be capped and labelled with the date the brine was generated and its salinity. Containers of HSB should be stored in the dark and maintained under room temperature until used.

7.3.7.6 If a source of HSB is available, test solutions can be made by following the directions below. Thoroughly mix together the deionized water and brine before mixing in the effluent.

7.3.7.7 Divide the salinity of the HSB by the expected test salinity to determine the proportion of deionized water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 25‰, 100‰ divided by 25‰ = 4.0. The proportion of brine is 1 part in 4 (one part brine to three parts deionized water).

7.3.7.8 To make 1 L of seawater at 25‰ salinity from a hypersaline brine of 100‰, 250 mL of brine and 750 mL of deionized water are required.

7.4 USE OF TAP WATER AS DILUTION WATER

7.4.1 The use of tap water as dilution water is discouraged unless it is dechlorinated and fully treated. Tap water can be dechlorinated by deionization, carbon filtration, or the use of sodium thiosulfate. Use of 3.6 mg/L (anhydrous) sodium thiosulfate will reduce 1.0 mg chlorine/L (APHA, 1992,

p. 4-36). Following dechlorination, total residual chlorine should not exceed 0.01 mg/L. Because of the possible toxicity of thiosulfate to test organisms, a control lacking thiosulfate should be included in toxicity tests utilizing thiosulfate-dechlorinated water.

7.4.2 To be adequate for general laboratory use following dechlorination, the tap water is passed through a deionizer and carbon filter to remove toxic metals and organics, and to control hardness and alkalinity.

7.5 **DILUTION WATER HOLDING**

7.5.1 A given batch of dilution water should not be used for more than 14 days following preparation because of the possible build-up of bacterial, fungal, or algal slime growth and the problems associated with it. The container should be kept covered and the contents should be protected from light.

SECTION 8

EFFLUENT AND RECEIVING WATER SAMPLING AND SAMPLE HANDLING

8.1 EFFLUENT SAMPLING

8.1.1 The effluent sampling point is ordinarily the same as that specified in the NPDES discharge permit (USEPA, 1979c). Conditions for exception would be: (1) better access to a sampling point between the final treatment and the discharge outfall; (2) if the effluent is chlorinated prior to discharge to the receiving waters, it may also be desirable to take samples prior to contact with the chlorine to determine toxicity of the unchlorinated effluent; or (3) in the event there is a desire to evaluate the toxicity of the influent to publicly owned treatment works or separate process waters in industrial facilities prior to their being combined with other process waters or non-contact cooling water, additional sampling points may be chosen.

8.1.2 The decision on whether to collect grab or composite samples is based on the requirements of the NPDES permit, the objectives of the test, and an understanding of the short and long-term operations and schedules of the discharger. If the effluent quality varies considerably with time, which can occur where holding times within the treatment facility are short, grab samples may seem preferable because of the ease of collection and the potential of observing peaks (spikes) in toxicity. However, the sampling duration of a grab sample is so short that full characterization of an effluent over a 24-h period would require a prohibitive number of separate samples and tests. Collection of a 24-h composite sample, however, may dilute toxicity spikes, and average the quality of the effluent over the sampling period. Sampling recommendations are provided below.

8.1.3 Aeration during collection and transfer of effluents should be minimized to reduce the loss of volatile chemicals.

8.1.4 Details of date, time, location, duration, and procedures used for effluent sample and dilution water collection should be recorded.

8.2 EFFLUENT SAMPLE TYPES

8.2.1 The advantages and disadvantages of effluent grab and composite samples are listed below:

8.2.1.1 Grab Samples

Advantages:

1. Easy to collect; require a minimum of equipment and on-site time.
2. Provide a measure of instantaneous toxicity. Toxicity spikes are not masked by dilution.

Disadvantages:

1. Samples are collected over a very short period of time and on a relatively infrequent basis. The chances of detecting a spike in toxicity would depend on the frequency of sampling, and the probability of missing spikes is high.

8.2.1.2 Composite Samples:

Advantages:

1. A single effluent sample is collected over a 24-h period.
2. The sample is collected over a much longer period of time than grab samples and contains all toxicity spikes.

Disadvantages:

1. Sampling equipment is more sophisticated and expensive, and must be placed on-site for at least 24 h.
2. Toxicity spikes may not be detected because they are masked by dilution with less toxic wastes.

8.3 EFFLUENT SAMPLING RECOMMENDATIONS

8.3.1 When tests are conducted on-site, test solutions can be renewed daily with freshly collected samples.

8.3.2 When tests are conducted off-site, samples are collected once, or daily, and used for test initiation and renewal.

8.3.3 Sufficient sample must be collected to perform the required toxicity and chemical tests. A 4-L (1-gal) CUBITAINER® will provide sufficient sample volume for most tests (see Tables 11-17).

8.3.4 The following effluent sampling methods are recommended:

8.3.4.1 Continuous Discharges

1. If the facility discharge is continuous, but the calculated retention time of the continuously discharged effluent is less than 14 days and the variability of the effluent toxicity is unknown, at a minimum, four grab samples or four composite samples are collected over a 24-h period. For example, a grab sample is taken every 6 h (total of four samples) and each sample is used for a separate toxicity test, or four successive 6-h composite samples are taken and each is used in a separate test.

2. If the calculated retention time of a continuously discharged effluent is greater than 14 days, or if it can be demonstrated that the wastewater does not vary more than 10% in toxicity over a 24-h period, regardless of retention time, a single grab sample is collected for a single toxicity test.

3. The retention time of the effluent in the wastewater treatment facility may be estimated from calculations based on the volume of the retention basin and rate of wastewater inflow. However, the calculated retention time may be much greater than the actual time because of short-circuiting in the holding basin. Where short-circuiting is suspected, or sedimentation may have reduced holding basin capacity, a more accurate estimate of the retention time can be obtained by carrying out a dye study.

8.3.4.2 Intermittent Discharges

8.3.4.2.1 If the facility discharge is intermittent, a grab sample is collected midway during each discharge period. Examples of intermittent discharges are:

1. When the effluent is continuously discharged during a single 8-h work shift (one sample is collected), or two successive 8-h work shifts (two samples are collected).

2. When the facility retains the wastewater during an 8-h work shift, and then treats and releases the wastewater as a batch discharge (one sample is collected).

3. When the facility discharges wastewater to an estuary only during an outgoing tide, usually during the 4 h following slack high tide (one sample is collected).

8.3.4.3 At the end of a shift, clean up activities may result in the discharge of a slug of toxic waste, which may require sampling and testing.

8.4 RECEIVING WATER SAMPLING

8.4.1 Logistical problems and difficulty in securing sampling equipment generally preclude the collection of composite receiving water samples for toxicity tests. Therefore, it is common practice to collect a single grab sample and use it throughout the test.

8.4.2 The sampling point is determined by the objectives of the test. In rivers, grab samples should be collected at mid-stream and mid-depth, if accessible. At estuarine and marine sites, samples are collected at mid-depth.

8.4.3 To determine the extent of the zone of toxicity in the receiving water downstream from the outfall, receiving water samples are collected at several distances downstream from the discharge. The time required for the effluent-receiving-water mixture to travel to sampling points downstream from the outfall, and the rate and degree of mixing, may be difficult to ascertain. Therefore, it may not be possible to correlate downstream toxicity with effluent toxicity at the discharge point unless a dye study is performed. The toxicity of receiving water samples from five stations downstream from the discharge point can be evaluated using the same number of test vessels and

test organisms as used in one effluent toxicity test with five effluent dilutions.

8.5 EFFLUENT AND RECEIVING WATER SAMPLE HANDLING, PRESERVATION, AND SHIPPING

8.5.1 Unless the samples are used in an on-site toxicity test the day of collection, it is recommended that they be held at 4°C until used to inhibit microbial degradation, chemical transformations, and loss of highly volatile toxic substances.

8.5.2 Composite samples should be chilled as they are collected. Grab samples should be chilled immediately following collection.

8.5.3 If the effluent has been chlorinated, total residual chlorine must be measured immediately following sample collection.

8.5.4 Sample holding time begins when the last grab sample in a series is taken (i.e., when a series of four grab samples are taken over a 24-h period), or when a 24-h composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time (holding time) from sample collection to first use of the sample in test initiation should not exceed 36 h. EPA believes that 36 h is adequate time to deliver the samples to the laboratories performing the tests in most cases. In the isolated cases, where the permittee can document that this delivery time cannot be met, the permitting authority can allow an option for on-site testing or a variance for an extension of shipped sample holding time. The request for a variance in sample holding time, directed to the EPA Regional Administrator under 40 CFR 136.3(e) must include supportive data which show that the toxicity of the effluent sample is not reduced (e.g., because of volatilization and/or sorption of toxics on the sample container surfaces) by extending the holding time beyond more than 36 h. However in no case should more than 72 h elapse between collection and first use of the sample. In static-renewal tests, the original sample may also be used to prepare test solution for renewal at 24 h, 48 h, and/or 72 h after test initiation, if stored at 4°C, with minimum head space, as described in Subsection 8.5. Guidance for determining the persistence of the sample is provided in Subsection 8.7.

8.5.5 To minimize the loss of toxicity due to volatilization of toxic constituents, all sample containers should be "completely" filled, leaving no air space between the contents and the lid.

8.5.6 SAMPLES USED IN ON-SITE TESTS

8.5.6.1 Samples collected for on-site tests should be used within 24 h.

8.5.7 SAMPLES SHIPPED TO OFF-SITE FACILITIES

8.5.7.1 Samples collected for off-site toxicity testing are to be chilled to 4°C during or immediately after collection, and shipped iced to the performing laboratory. Sufficient ice should be placed with the sample in the shipping container to ensure that ice will still be present when the sample arrives at

test organisms as used in one effluent toxicity test with five effluent dilutions.

8.5 EFFLUENT AND RECEIVING WATER SAMPLE HANDLING, PRESERVATION, AND SHIPPING

8.5.1 Unless the samples are used in an on-site toxicity test the day of collection, it is recommended that they be held at 4°C until used to inhibit microbial degradation, chemical transformations, and loss of highly volatile toxic substances.

8.5.2 Composite samples should be chilled as they are collected. Grab samples should be chilled immediately following collection.

8.5.3 If the effluent has been chlorinated, total residual chlorine must be measured immediately following sample collection.

8.5.4 Sample holding time begins when the last grab sample in a series is taken (i.e., when a series of four grab samples are taken over a 24-h period), or when a 24-h composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time (holding time) from sample collection to first use of the sample in test initiation should not exceed 36 h. EPA believes that 36 h is adequate time to deliver the samples to the laboratories performing the tests in most cases. In the isolated cases, where the permittee can document that this delivery time cannot be met, the permitting authority can allow an option for on-site testing or a variance for an extension of shipped sample holding time. The request for a variance in sample holding time, directed to the EPA Regional Administrator under 40 CFR 136.3(e) must include supportive data which show that the toxicity of the effluent sample is not reduced (e.g., because of volatilization and/or sorption of toxics on the sample container surfaces) by extending the holding time beyond more than 36 h. However in no case should more than 72 h elapse between collection and first use of the sample. In static-renewal tests, the original sample may also be used to prepare test solution for renewal at 24 h, 48 h, and/or 72 h after test initiation, if stored at 4°C, with minimum head space, as described in Subsection 8.5. Guidance for determining the persistence of the sample is provided in Subsection 8.7.

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the laboratory and is unpacked. Insulating material must not be placed between the ice and the sample in the shipping container.

8.5.7.2 Samples may be shipped in one or more 4-L (1-gal) CUBITAINERS® or new plastic "milk" jugs. All sample containers should be rinsed with source water before being filled with sample. After use with receiving water or effluents, CUBITAINERS® and plastic jugs are punctured to prevent reuse.

8.5.7.3 Several sample shipping options are available, including Express Mail, air express, bus, and courier service. Express Mail is delivered seven days a week. Saturday and Sunday shipping and receiving schedules of private carriers vary with the carrier.

8.6 SAMPLE RECEIVING

8.6.1 Upon arrival at the laboratory, samples are logged in and the temperature is measured and recorded. If the samples are not immediately prepared for testing, they are stored at 4°C until used.

8.6.2 Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory, and the sample holding time should not exceed 36 h before first use unless a variance has been granted by the NPDES permitting authority.

8.7 PERSISTENCE OF EFFLUENT TOXICITY DURING SAMPLE SHIPMENT AND HOLDING

8.7.1 The persistence of the toxicity of an effluent prior to its use in a toxicity test is of interest in assessing the validity of toxicity test data, and in determining the possible effects of allowing an extension of the holding time. Where a variance in holding time (>36 h, but ≤72 h) is requested by a permittee (see Subsection 8.5.4 above), information on the effects of the extension in holding time on the toxicity of the samples must be obtained by comparing the results of multi-concentration acute toxicity tests performed on effluent samples held 36 h with toxicity test results using the same samples after they were held for the requested, longer period. The portion of the sample set aside for the second test must be held under the same conditions as during shipment and holding.

SECTION 9

ACUTE TOXICITY TEST PROCEDURES

9.1 PREPARATION OF EFFLUENT AND RECEIVING WATER SAMPLES FOR TOXICITY TESTS

9.1.1 When aliquots are removed from the sample container, the head space above the remaining sample should be held to a minimum. Air which enters a container upon removal of sample should be expelled by compressing the container before reclosing, if possible (i.e., where a CUBITAINER® used), or by using an appropriate discharge valve (spigot).

9.1.2 It may be necessary to first coarse-filter samples through a sieve having 2- to 4-mm mesh openings to remove debris and/or break up large floating or suspended solids. If samples contain indigenous organisms that may attack or be confused with the test organisms, the samples must be filtered through a sieve with 60 μm mesh openings. **Caution**: filtration may remove some toxicity.

9.1.3 At a minimum, pH, conductivity or salinity, and total residual chlorine are measured in the undiluted effluent or receiving water, and pH and conductivity are measured in the dilution water.

9.1.4 It is recommended that total alkalinity and total hardness also be measured in the undiluted test water (effluent or receiving water) and the dilution water.

9.1.5 Total ammonia is measured in effluent and receiving water samples where toxicity may be contributed by unionized ammonia (i.e., where total ammonia >5 mg/L). The concentration (mg/L) of unionized (free) ammonia in a sample is a function of temperature and pH, and is calculated using the percentage value obtained from Table 9, under the appropriate pH and temperature, and multiplying it by the concentration (mg/L) of total ammonia in the sample.

9.1.6 Effluents and receiving waters can be dechlorinated using 6.7 mg/L anhydrous sodium thiosulfate to reduce 1 mg/L chlorine (Standard Methods, 18th Edition, APHA, 1992, p. 9-32; note that the amount of thiosulfate required to dechlorinate effluents is greater than the amount needed to dechlorinate tap water). Since thiosulfate may contribute to sample toxicity, a thiosulfate control should be used in the test in addition to the normal dilution water control.

9.1.7 The DO concentration in the samples should be near saturation prior to use. Aeration will bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH. However, aeration during collection, transfer, and preparation of samples should be minimized to reduce the loss of volatile chemicals.

9.1.8 If the samples must be warmed to bring them to the prescribed test temperature, supersaturation of the dissolved oxygen and nitrogen may become a problem. To avoid this problem, the effluent and dilution water are checked

TABLE 9. PERCENT UNIONIZED NH_3 IN AQUEOUS AMMONIA SOLUTIONS: TEMPERATURES 15-26°C AND PH's 6.0-8.9[1]

pH	TEMPERATURE (°C)											
	15	16	17	18	19	20	21	22	23	24	25	26
6.0	0.0274	0.0295	0.0318	0.0343	0.0369	0.0397	427	0.0459	0.0493	0.0530	0.0568	0.0610
6.1	0.0345	0.0372	0.0400	0.0431	0.0464	0.0500	537	0.0578	0.0621	0.0667	0.0716	0.0768
6.2	0.0434	0.0468	0.0504	0.0543	0.0584	0.0629	676	0.0727	0.0781	0.0901	0.0901	0.0966
6.3	0.0546	0.0589	0.0634	0.0683	0.0736	0.0792	851	0.0915	0.0983	0.1134	0.1134	0.1216
6.4	0.0687	0.0741	0.0799	0.0860	0.0926	0.0996	07	0.115	0.124	0.133	0.143	0.153
6.5	0.0865	0.0933	0.1005	0.1083	0.1166	0.1254	35	0.145	0.156	0.167	0.180	0.193
6.6	0.109	0.117	0.127	0.136	0.147	0.158	70	0.182	0.196	0.210	0.226	0.242
6.7	0.137	0.148	0.159	0.171	0.185	0.199	14	0.230	0.247	0.265	0.284	0.305
6.8	0.172	0.186	0.200	0.216	0.232	0.250	69	0.289	0.310	0.333	0.358	0.384
6.9	0.217	0.234	0.252	0.271	0.292	0.314	38	0.363	0.390	0.419	0.450	0.482
7.0	0.273	0.294	0.317	0.342	0.368	0.396	25	0.457	0.491	0.527	0.566	0.607
7.1	0.343	0.370	0.399	0.430	0.462	0.497	35	0.575	0.617	0.663	0.711	0.762
7.2	0.432	0.466	0.502	0.540	0.581	0.625	72	0.722	0.776	0.833	0.893	0.958
7.3	0.543	0.586	0.631	0.679	0.731	0.786	45	0.908	0.975	1.05	1.12	1.20
7.4	0.683	0.736	0.793	0.854	0.918	0.988	61	1.140	1.224	1.31	1.41	1.51
7.5	0.858	0.925	0.996	1.07	1.15	1.24	3	1.43	1.54	1.65	1.77	1.89
7.6	1.08	1.16	1.25	1.35	1.45	1.56	7	1.80	1.93	2.07	2.21	2.37
7.7	1.35	1.46	1.57	1.69	1.82	1.95	0	2.25	2.41	2.59	2.77	2.97
7.8	1.70	1.83	1.97	2.12	2.28	2.44	2	2.82	3.02	3.24	3.46	3.71
7.9	2.13	2.29	2.46	2.65	2.85	3.06	8	3.52	3.77	4.04	4.32	4.62
8.0	2.66	2.87	3.08	3.31	3.56	3.82	0	4.39	4.70	5.03	5.38	5.75
8.1	3.33	3.58	3.85	4.14	4.44	4.76	0	5.46	5.85	6.25	6.68	7.14
8.2	4.16	4.47	4.80	5.15	5.52	5.92	4	6.78	7.25	7.75	8.27	8.82
8.3	5.18	5.56	5.97	6.40	6.86	7.34	5	8.39	8.96	9.56	10.2	10.9
8.4	6.43	6.90	7.40	7.93	8.48	9.07	9	10.3	11.0	11.7	12.5	13.3
8.5	7.97	8.54	9.14	9.78	10.45	11.16	0	12.7	13.5	14.4	15.2	16.2
8.6	9.83	10.5	11.2	12.0	12.8	13.6		15.5	16.4	17.4	18.5	19.5
8.7	12.07	12.9	13.8	14.7	15.6	16.6		18.7	19.8	21.0	22.2	23.4
8.8	14.7	15.7	16.7	17.8	18.9	20.0		22.5	23.7	25.1	26.4	27.8
8.9	17.9	19.0	20.2	21.4	22.7	24.0		26.7	28.2	29.6	31.1	32.6

[1]Table provided by Teresa Norberg-King, Environmental Research Laboratory, Duluth, Minnesota. Also see Emerson, et. al., 1975, Thurston, et. al., 1974, and USEPA, 1985.

with a DO probe after reaching test temperature and, if the DO is greater than 100% saturation or lower than 4.0 mg/L, the solutions are aerated moderately (approximately 500 mL/min) for a few minutes, using an airstone, until the DO is within the prescribed range (≥4.0 mg/L when using warm water species, or ≥6.0 mg/L when using cold water species). Caution: avoid excessive aeration.

9.1.9 Mortality due to pH alone may occur if the pH of the sample falls outside the range of 6.0-9.0. Thus, the presence of other forms of toxicity (metals and organics) in the sample may be masked by the toxic effects of low or high pH. The question about the presence of other toxicants can be answered only by performing two parallel tests, one with an adjusted pH, and one without an adjusted pH. Freshwater samples are adjusted to pH 7.0, and marine samples are adjusted to pH 8.0, by adding 1N NaOH or 1N HCl dropwise, as required, being careful to avoid overadjustment.

9.2 PRELIMINARY TOXICITY RANGE-FINDING TESTS

9.2.1 USEPA Regional and State personnel generally have observed that it is not necessary to conduct a toxicity range-finding test prior to initiating a static, acute, definitive toxicity test. However, when preparing to perform a static test with an sample of completely unknown quality, or before initiating a flow-through test, it is advisable to conduct a preliminary toxicity range-finding test.

9.2.2 A toxicity range-finding test ordinarily consists of a down-scaled, abbreviated static acute test in which groups of five organisms are exposed to several widely-spaced sample dilutions in a logarithmic series, such as 100%, 10.0%, 1.00%, and 0.100%, and a control, for 8-24 h. **Caution:** if the sample must also be used for the full-scale definitive test, the 36-h limit on holding time (Section 8, Effluent and Receiving Water Sampling and Sample Handling, Subsection 8.5.4) must not be exceeded before the definitive test is initiated.

9.2.3 It should be noted that the toxicity (LC50) of a sample observed in a range-finding test may be significantly different from the toxicity observed in the follow-up definitive test because: (1) the definitive test is usually longer; and (2) the test may be performed with a sample collected at a different time, and possibly differing significantly in the level of toxicity.

9.3 MULTI-CONCENTRATION (DEFINITIVE) EFFLUENT TOXICITY TESTS

9.3.1 The tests recommended for use in determining discharge permit compliance in the NPDES program are multi-concentration, or definitive, tests which provide (1) a point estimate of effluent toxicity in terms of a LC50, or (2) a No-Observed-Adverse-Effect Concentration (NOAEC) defined in terms of mortality, and obtained by hypothesis testing. The tests may be static non-renewal, static renewal, or flow-through.

9.3.2 The tests consist of a control and a minimum of five effluent concentrations commonly selected to approximate a geometric series, such as 100%, 50%, 25%, 12.5%, and 6.25%, by using a dilution factor of 0.5.

9.3.3 These tests are also to be used in determining compliance with permit limits on the mortality of the "instream" or receiving water concentration (RWC) of effluents by bracketing the RWC with effluent concentrations in the following manner: (1) 100% effluent, (2) [RWC + 100]/2, (3) RWC, (4) RWC/2, and (5) RWC/4. For example, where the RWC = 50%, the effluent concentrations used in the test would be 100%, 75%, 50%, 25%, and 12.5%.

9.3.4 If acute/chronic ratios are to be determined by simultaneous acute and short-term chronic tests with a single species, using the same sample, both types of tests must use the same test conditions, i.e., temperature, water hardness, salinity, etc.

9.4 **RECEIVING WATER TESTS**

9.4.1 Receiving water toxicity tests generally consist of 100% receiving water and a control. The total hardness or salinity of the control should be comparable to the receiving water.

9.4.2 The data from the two treatments are analyzed by hypothesis testing to determine if test organism survival in the receiving water differs significantly from the control. A minimum of four replicates and 10 organisms per replicate are required for each treatment (see Tables 11-17).

9.4.3 In cases where the objective of the test is to estimate the degree of toxicity of the receiving water, a definitive, multi-concentration test is performed by preparing dilutions of the receiving water, using a ≥0.5 dilution series, with a suitable control water.

9.5 **STATIC TESTS**

9.5.1 Static tests may be non-renewal or renewal.

9.5.2 An excess volume of each dilution is prepared to provide sufficient material for toxicity testing and routine chemical analyses. The solutions are well mixed with a glass rod, TEFLON® stir bar, or other means. Aliquots of each sample concentration are delivered to the test chambers, and the chambers are arranged in random order. The test solutions are brought to the required temperature, and the test organisms are added. The remaining volumes of each sample concentration are used, as necessary, for the chemical analyses.

9.5.3 Saline dilution water can be prepared by adding dry salts (FORTY FATHOMS® or equivalent, or modified GP2) or hypersaline brine to de-ionized water, or a suitable surface freshwater, to adjust the salinity of the entire dilution series. If saline receiving water is used as the diluent, a salinity control must be prepared using deionized water and dried sea salts to determine if the addition of sea salts alone has an adverse effect on the test organisms. It may be desirable to conduct static toxicity tests at several salinities.

9.5.4 If the effluent has low salinity, but the test is to be conducted with a salt water organism, the test solutions may be prepared by adding dry ocean

salts or hypersaline brine to a sufficient quantity of 100% effluent to raise the salinity to the required level, which will depend on the objectives of the test and the policy of the regulatory agency. After the addition of the dried salts, stir gently for 30 to 60 min, preferably with a magnetic stirrer, to ensure that the salts are in solution. It is important to check the final salinity with a refractometer.

9.5.5 Addition of dry salts to effluents and dilution water may change the pH and affect the toxicity of the waste. If the objective of the test is to determine the toxicity of the effluent at the original pH, the pH of the salinity-adjusted solutions can be brought to the required level by dropwise addition of 1N HCl or 1N NaOH. It is recommended that a concurrent test be conducted with salinity-adjusted effluent in which the pH has not been altered after adding the salt.

9.5.6 The volume of the effluent used must be sufficient to prepare all percent concentrations of the effluent needed for the toxicity test and for routine chemical analysis. For example, to conduct tests with *Menidia*, the use of 200 mL of test solution in each of duplicate exposure vessels and five concentrations of effluent (10 exposure vessels), would require a total of 1 L of 100% effluent. However, to provide sufficient volumes of test solutions for routine chemical analysis and for toxicity testing, additional effluent would be required (1.5-2.0 L).

9.5.7 A standard control lacking thiosulfate should be included in tests where the dilution water was prepared by dechlorinating tap water with thiosulfate.

9.5.8 If, within 1 h of the start of the test, 100% mortality has occurred in the higher effluent concentrations (such as 100% and 50%), additional concentrations of effluents, such as 3.1%, 1.6%, and 0.8%, are added to the test at the lower end of the concentration series.

9.5.9 Increases in pH may occur in test solutions during acute, static non-renewal toxicity of pollutants such as ammonia. This problem can be avoided by conducting a test in a static renewal or flow through mode rather than a static non-renewal mode.

9.6 FLOW-THROUGH TESTS

9.6.1 Flow-through tests are usually performed with the same effluent concentrations that are used for static tests, except that where the receiving water is saline and the effluent is not, 100% effluent cannot be tested with a marine organism. Examples of flow-through test systems are provided in the Appendix. Small organisms, such as mysids and daphnids, are confined in screened enclosures placed in the flow-through chambers. More than one species may be used in the same test chamber in a given test, if segregated.

9.6.2 The dilutor system should be operated long enough prior to adding the test organisms to calibrate the dilutor and make the necessary adjustments in the temperature, flow rate through the test chambers, and aeration. The flow rate through the proportional dilutor must provide for a minimum of five 90%

replacements of water volume in each test chamber every 24 h (see Figure 2). This replacement rate should provide sufficient flow to maintain an adequate concentration of dissolved oxygen. The dilutor should also be capable of maintaining the test concentration at each dilution within 5% of the starting concentration for the duration of the test. The calibration of the dilutor should be checked carefully before the test begins to determine the volume of effluent and dilution water used in each portion of the effluent delivery system and the flow rate through each test chamber. The general operation of the dilutor should be checked at least at the beginning and end of each day during the test.

9.6.3 The control consists of the same dilution water, test conditions, procedures, and organisms used in testing the effluent. In the event a test is to be conducted with salt water organisms, where each effluent dilution has a different salinity, a static control is prepared for the lowest (or highest, in the case of high salinity, e.g. brine wastes) salinity level used in the flow-through test to determine if salinity alone has any adverse effects on the test organisms.

9.7 NUMBER OF TEST ORGANISMS

9.7.1 A minimum of 20 organisms of a given species are exposed to each effluent concentration (Jensen, 1972). Small fish and invertebrates are captured with 4- to 8-mm inside diameter pipettes. Organisms larger than 10-mm can be captured by dip net. In a typical toxicity test involving five effluent concentrations and a control (six concentrations X 20 organisms per concentration), fish and other large test organisms are captured from a common pool and distributed sequentially to the test chambers until the required number of organisms are placed in each. The test chambers are then positioned randomly. To avoid carryover of excess culture water in transferring small organisms to the test chambers, it may be advantageous to distribute small organisms, such as daphnids, mysids, and larval fish, first to small holding vessels, such as weighing boats, petri dishes, or small beakers. The water in the intermediary holding vessels is then drawn down to a small volume and the entire lot is transferred to a test chamber. In the case of daphnids, both excessive handling and carryover of culture water can be avoided by placing the tip of the transfer pipettes below the surface of the water in the test chambers and allowing the organisms to swim out of the pipettes without discharging the contents.

9.8 REPLICATE TEST CHAMBERS

9.8.1 Two or more test chambers are provided for each effluent concentration and the control. Although the data from duplicate chambers are usually combined to determine the LC50 and confidence interval, the practice of dividing the test population for each effluent concentration between two or more replicate chambers has several advantages and is considered good laboratory practice because it: (1) permits easier viewing and counting of test organisms; (2) more easily avoids possible violations of loading limits, which might occur if all of the test organisms are placed in a single test vessel; and (3) ensures against the invalidation of the test which might

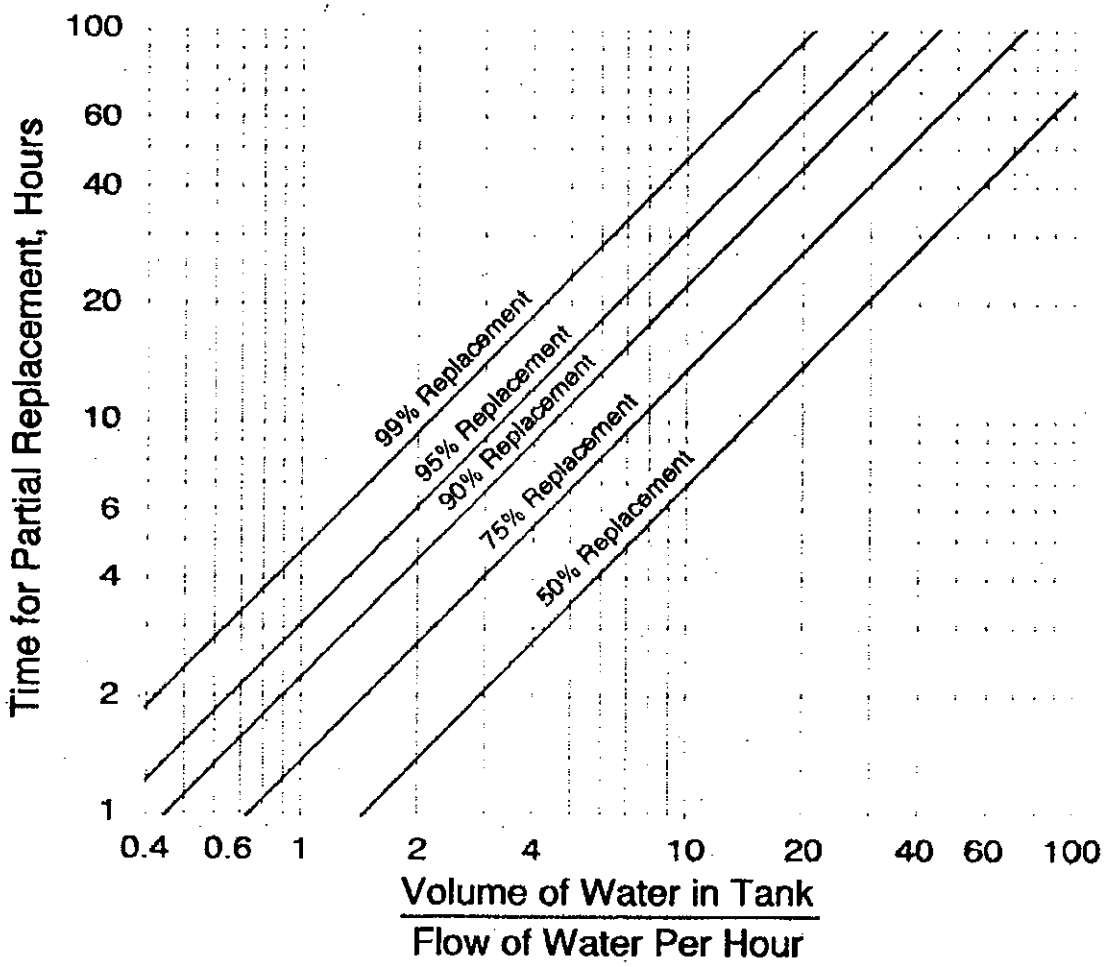

Figure 2. Approximate times required to replace water in test chambers in flow-through tests. For example: for a chamber containing 4 L, with a flow of 2 L/h, the above graph indicates that 90% of the water would be replaced every 4.8 h. The same time period (such as hours) must be used on both axes, and the same unit of volume (such as liters) must be used for both volume and flow (From: Sprague, 1969).

result from accidental loss of a test vessel, where all of the test organisms for a given treatment are in a single chamber.

9.9 LOADING OF TEST ORGANISMS

9.9.1 A limit is placed on the loading (weight) of organisms per liter of test solution to minimize the depletion of dissolved oxygen, the accumulation of injurious concentrations of metabolic waste products, and/or stress induced by crowding, any of which could significantly affect the test results. However, the probability of exceeding loading limits is greatly reduced with the use of very young test organisms.

9.9.2 For both renewal and non-renewal static tests, loading in the test solutions must not exceed the following live weights: 1.1 g/L at 15°C, 0.65 g/L at 20°C, or 0.40 g/L at 25°C.

9.9.3 For flow-through tests, the live weight of test organisms in the test chambers must not exceed 7.0 g/L of test solution at 15°C, or 2.5 g/L at 25°C.

9.10 ILLUMINATION

9.10.1 Light of the quality and intensity normally obtained in the laboratory during working hours is adequate (10-20 $\mu E/m^2/s$ or 50-100 ft-c). A uniform photoperiod of 16 h light and 8 h darkness can be achieved in the laboratory or environmental chamber, using automatic timers.

9.11 FEEDING

9.11.1 Where indicated in the test summary tables (Tables 11-17), food is made available to test organisms while holding before they are placed in the test chambers. The organisms are fed at test renewal, 48 h after the test is initiated, if Regional or State policy requires a 96-h test duration.

9.11.2 Where *Artemia* nauplii are fed, the nauplii are first concentrated on a NITEX® screen and then are resuspended in fresh or salt water, depending on the salinity of the test solutions, using just enough water to form a slurry that can be transferred by pipette. It should be noted that *Artemia* nauplii placed in freshwater usually die in 4 h, generally are not eaten after death, and decay rapidly, whereas those placed in saline water remain viable and can serve as food for the duration of the test.

9.11.3 Problems caused by feeding, such as the possible alteration of the toxicant concentration, the build-up of food and metabolic wastes and resulting oxygen demand, are common in static test systems. Where feeding is necessary, excess food should be removed daily by aspirating with a pipette.

9.11.4 Feeding does not cause the above problems in flow-through systems. However, it is advisable to remove excess food, fecal material, and any particulate matter that settles from the effluent, from the bottom of the test vessels daily by aspirating with a pipette.

9.12 TEST TEMPERATURE

9.12.1 Test temperature will depend on the test species and objectives of the test (see Tables 11-17). Where acute and short-term chronic toxicity tests are performed simultaneously with the same species to determine acute:chronic ratios, both tests must be performed at the chronic test temperature. The average daily temperature of the test solutions must be maintained within ± 1°C of the selected test temperature, for the duration of the test. This can be accomplished for static tests by use of a water bath or environmental chamber, and in flow-through tests by passing the effluent and/or dilution water through separate coils immersed in a heating or cooling water bath prior to entering the dilutor system. Coils should be made from materials recommended in Section 5, Facilities and Equipment.

9.13 STRESS

9.13.1 Minimize stress on test organisms by avoiding unnecessary disturbances.

9.14 DISSOLVED OXYGEN CONCENTRATION

9.14.1 Aeration during the test may alter the results and should be used only as a last resort to maintain the required DO. Aeration can reduce the apparent toxicity of the test solutions by stripping them of highly volatile toxic substances, or increase its toxicity by altering the pH. However, the DO in the test solution must not be permitted to fall below 4.0 mg/L for warm water species and 6.0 mg/L for cold water species. Oxygen saturation values in fresh and saline waters can be determined from Figure 3 and Table 10, respectively.

9.14.2 In static tests, low DOs commonly occur in the higher concentrations of wastewater. Aeration is accomplished by bubbling air through a pipet at the rate of 100 bubbles/min. If aeration is necessary, <u>all</u> test solutions must be aerated. It is advisable to monitor the DO closely during the first few hours of the test. Samples with a potential DO problem generally show a downward trend in DO within 4 to 8 h after the test is started. Unless aeration is initiated during the first 8 h of the test, the DO may be exhausted during an unattended period, thereby invalidating the test.

9.14.3 In most flow-through tests, DO depletion is not a problem in the test chambers because aeration occurs as the liquids pass through the dilutor system. If the DO decreases to a level that would be a source of additional stress, the turnover rate of the solutions in the test chambers must be increased sufficiently to maintain acceptable DO levels. If the increased turnover rate does not maintain adequate DO levels, aerate the dilution water prior to the addition of the effluent, and aerate all test solutions. To reduce the potential for driving off volatile compounds in the wastewater, aeration may be accomplished by bubbling air through a 1-mL pipet at a rate of no more than 100 bubbles/min, using an air valve to control the flow.

9.14.4 Caution must be exercised to avoid excessive aeration. Turbulence caused by aeration should not result in a physical stress to the test

TABLE 10. OXYGEN SOLUBILITY (MG/L) IN WATER AT EQUILIBRIUM WITH AIR AT 760 MM HG (AFTER RICHARDS AND CORWIN, 1956)

TEMP	SALINITY (‰)									
°C	0	5	10	15	20	25	30	35	40	43
0	14.2	13.8	13.4	12.9	12.5	12.1	11.7	11.2	10.8	10.6
1	13.8	13.4	13.0	12.6	12.2	11.8	11.4	11.0	10.6	10.3
2	13.4	13.0	12.6	12.2	11.9	11.5	11.1	10.7	10.3	10.0
3	13.1	12.7	12.3	11.9	11.6	11.2	10.8	10.4	10.0	9.8
4	12.7	12.3	12.0	11.6	11.3	10.9	10.5	10.1	9.8	9.5
5	12.4	12.0	11.7	11.3	11.0	10.6	10.2	9.8	9.5	9.3
6	12.1	11.7	11.4	11.0	10.7	10.3	10.0	9.6	9.3	9.1
8	11.5	11.2	10.8	10.5	10.2	9.8	9.5	9.2	8.9	8.7
10	10.9	10.7	10.3	10.0	9.7	9.4	9.1	8.8	8.5	8.3
12	10.5	10.2	9.9	9.6	9.3	9.0	8.7	8.4	8.1	7.9
14	10.0	9.7	9.5	9.2	8.9	8.6	8.3	8.1	7.8	7.6
16	9.6	9.3	9.1	8.8	8.5	8.3	8.0	7.7	7.5	7.3
18	9.2	9.0	8.7	8.5	8.2	8.0	7.7	7.5	7.2	7.1
20	8.9	8.6	8.4	8.1	7.9	7.7	7.4	7.2	6.9	6.8
22	8.6	8.4	8.1	7.9	7.6	7.4	7.2	6.9	6.7	6.6
24	8.3	8.1	7.8	7.6	7.4	7.2	6.9	6.7	6.5	6.4
26	8.1	7.8	7.6	7.4	7.2	7.0	6.7	6.5	6.3	6.1
28	7.8	7.6	7.4	7.2	7.0	6.8	6.5	6.3	6.1	6.0
30	7.6	7.4	7.1	6.9	6.7	6.5	6.3	6.1	5.9	5.8
32	7.3	7.1	6.9	6.7	6.5	6.3	6.1	5.9	5.7	5.6

CORRECTION FACTORS FOR OXYGEN SATURATION AT VARIOUS ALTITUDES

ALTITUDE		PRESSURE	
FT	M	MM	FACTOR
0	0	760	1.00
330	100	750	1.01
665	200	741	1.03
980	300	732	1.04
1310	400	723	1.05
1640	500	714	1.06
1970	600	705	1.08
2300	700	696	1.09
2630	800	687	1.11
2950	900	679	1.12
3280	1000	671	1.13
3610	1100	663	1.15
3940	1200	655	1.16
4270	1300	647	1.17
4600	1400	639	1.19
4930	1500	631	1.20
5250	1600	623	1.22
5580	1700	615	1.24
5910	1800	608	1.25
6240	1900	601	1.26
6560	2000	594	1.28
6900	2100	587	1.30
7220	2200	580	1.31
7550	2300	573	1.33
7880	2400	566	1.34
8200	2500	560	1.36

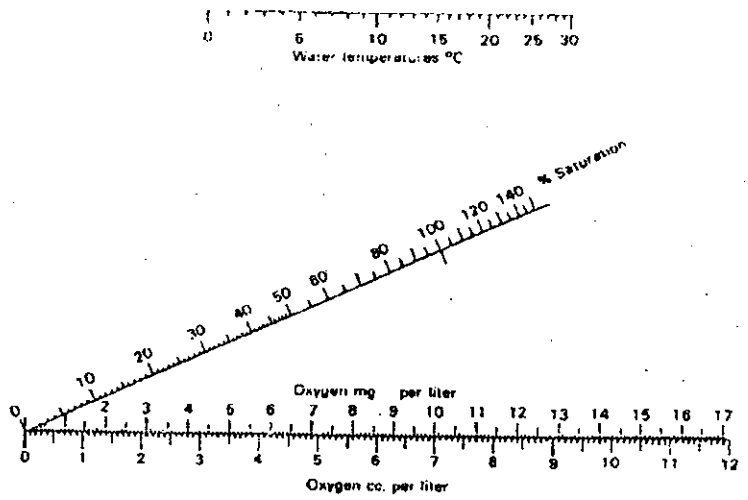

Figure 3. Rawson's nomograph for obtaining oxygen saturation values in freshwater at different temperatures at sea level. When a straightedge is used to connect the water temperature on the upper scale and the concentration on the lower scale, the percent saturation can be read from the point of intersection on the diagonal scale. To determine the percent saturation at locations above sea level, factors are provided to convert oxygen concentrations measured at various altitudes to sea level values in the table at the upper left. For example, an oxygen concentration of 6.4 mg/L measured in a body of water at an altitude of 1000 m and a temperature of 15°C would be equivalent to a concentration of 6.4 x 1.13, or 7.2 mg/L, at sea level. To determine the percent saturation, a straightedge is used to connect the point at 15°C on the temperature scale with the point, 7.2 mg/L on the concentration scale, and the percent saturation is read at the point of intersection (68%) on the diagonal scale. (From Welch, 1948).

organisms. When aeration is used, the methodology must be detailed in the report. For safety reasons, pure oxygen should not be used to aerate test solutions.

9.15 TEST DURATION

9.15.1 Test duration may vary from 24 to 96 h depending on the objectives of the test and the requirements of the regulatory authority. For specific information on test duration, see the tables summarizing the test conditions below.

9.16 ACCEPTABILITY OF TEST RESULTS

9.16.1 For the test results to be acceptable, survival in controls must be at least 90%. Tests in which the control survival is less than 90% are invalid, and must be repeated. In tests with specific chemicals, the concentration of the test material must not vary more than 20% at any treatment level during the exposure period.

9.16.2 Upon subsequent completion of a valid test, the results of all tests, valid and invalid, are reported to the regulatory authority with an explanation of the tests performed and results.

9.17 SUMMARY OF TEST CONDITIONS FOR THE PRINCIPAL TEST ORGANISMS

9.17.1 Summaries of the test conditions for the daphnids, *Ceriodaphnia dubia*, *Daphnia pulex*, and *D. magna*, fathead minnows, *Pimephales promelas*, rainbow trout, *Oncorhynchus mykiss*, brook trout, *Salvelinus fontinalis*, the mysid, *Mysidopsis bahia*, sheepshead minnows, *Cyprinodon variegatus*, and silversides, *Menidia beryllina*, *M. menidia*, and *M. peninsulae*, are provided in Tables 11-17.

TABLE 11. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *CERIODAPHNIA DUBIA* ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal, static-renewal, or flow-through
2. Test duration:	24, 48, or 96 h
3. Temperature:[1]	20°C ± 1°C; or 25°C ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	30 mL (minimum)
8. Test solution volume:	15 mL (minimum)
9. Renewal of test solutions:	Minimum, after 48 h
10. Age of test organisms:	Less than 24-h old
11. No. organisms per test chamber:	Minimum, 5 for effluent and receiving water tests
12. No. replicate chambers per concentration:	Minimum, 4 for effluent and receiving water tests
13. No. organisms per concentration:	Minimum, 20 for effluent and receiving water tests

[1]Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and water hardness.

TABLE 11. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *CERIODAPHNIA DUBIA* ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

14. Feeding regime:	Feed YCT and *Selenastrum* while holding prior to the test; newly-released young should have food available a minimum of 2 h prior to use in a test; add 0.1 mL each of YCT and *Selenastrum* 2h prior to test solution renewal at 48 h
15. Test chamber cleaning:	Cleaning not required
16. Test chamber aeration:	None
17. Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% OMW (see Section 7 Dilution Water), receiving water,ground water, or synthetic water, modified to reflect receiving water hardness.
18. Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19. Dilution series:	Effluents: ≥ 0.5 dilution series Receiving Waters: None, or ≥ 0.5 dilution series
20. Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (Significant difference from control)
21. Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period.
22. Sample volume required:	1 L
23. Test acceptability criterion:	90% or greater survival in controls

TABLE 12. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *DAPHNIA PULEX* AND *D. MAGNA* ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal, static-renewal, or flow-through
2. Test duration:	24, 48, or 96 h
3. Temperature:[1]	20°C ± 1°C; or 25°C ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	30 mL (minimum)
8. Test solution volume:	25 mL (minimum)
9. Renewal of test solutions:	Minimum, after 48 h
10. Age of test organisms:	Less than 24-h old
11. No. organisms per test chamber:	Minimum, 5 for effluent and receiving water tests
12. No. replicate chambers per concentration:	Minimum, 4 for effluent and receiving water tests
13. No. organisms per concentration:	Minimum, 20 for effluent and receiving water tests
14. Feeding regime:	Feed YCT and *Selenastrum* while holding prior to the test; newly-released young should have food available a minimum of 2 h prior to use in a test; add 0.1 mL each of YCT and *Selenastrum* 2 h prior to test solution renewal at 48 h

[1]Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and water hardness.

TABLE 12. SUMMARY OF CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *DAPHNIA PULEX* AND *D. MAGNA* ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

15. Test chamber cleaning:	Cleaning not required
16. Test chamber aeration:	None
17. Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7, Dilution Water), receiving water, ground water, or synthetic water, modified to reflect receiving water hardness.
18. Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19. Dilution series:	Effluents: ≥0.5 dilution series Receiving Waters: None, or ≥0.5 dilution series
20. Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (Significant difference from control)
21. Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period.
22. Sample volume required:	1 L
23. Test acceptability criterion:	90% or greater survival in controls

TABLE 13. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through
2. Test duration:	24, 48, or 96 h
3. Temperature:[2]	20°C ± 1°C; or 25°C ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	250 mL (minimum)
8. Test solution volume:	200 mL (minimum)
9. Renewal of test solutions:	Minimum, after 48 h
10. Age of test organisms:	1-14 days; 24-h range in age
11. No. organisms per test chamber:	Minimum, 10 for effluent and receiving water tests
12. No. replicate chambers per concentration:	Minimum, 2 for effluent tests Minimum, 4 for receiving water tests
13. No. organisms per concentration:	Minimum, 20 for effluent tests Minimum, 40 for receiving water tests
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h

[1] *Cyprinella leedsi* (Bannerfin shiner, formerly *Notropis leedsi*) can be used with the test conditions in this table, where it is the required test organism in discharge permits.

[2] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and water hardness.

TABLE 13. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7, Dilution Water), receiving water, ground water, or synthetic water, modified to reflect receiving water hardness.
18. Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19. Dilution series:	Effluents: ≥0.5 dilution series Receiving Waters: None, or ≥0.5 dilution series
20. Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (Significant difference from control)
21. Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period.
22. Sample volume required:	2 L for effluents and receiving waters
23. Test acceptability criterion:	90% or greater survival in controls

TABLE 14. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, AND BROOK TROUT, *SALVELINUS FONTINALIS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal, static-renewal, or flow-through
2. Test duration:	24, 48, or 96 h
3. Temperature:	12°C ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness. Light intensity should be raised gradually over a 15 min period at the beginning of the photoperiod, and lowered gradually at the end of the photoperiod, using a dimmer switch or other suitable device.
7. Test chamber size:	5 L (minimum) (test chambers should be covered to prevent fish from jumping out)
8. Test solution volume:	4 L (minimum)
9. Renewal of test solutions:	Minimum, after 48 h
10. Age of test organisms:	Rainbow Trout: 15-30 days (after yolk sac absorption to 30 days) Brook Trout: 30-60 days
11. No. organisms per test chamber:	Minimum, 10 for effluent and receiving water tests
12. No. replicate chambers per concentration:	Minimum, 2 for effluent tests Minimum, 4 for receiving water tests
13. No. organisms per concentration:	Minimum, 20 for effluent tests Minimum, 40 for receiving water tests
14. Feeding regime:	Feeding not required
15. Test chamber cleaning:	Cleaning not required

TABLE 14. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, AND BROOK TROUT, *SALVELINUS FONTINALIS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

16. Test solution aeration:	None, unless DO concentration falls below 6.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7, Dilution Water), receiving water, ground water, or synthetic water, modified to reflect receiving water hardness.
18. Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19. Dilution series:	Effluents: ≥0.5 dilution series Receiving Waters: None, or ≥0.5 dilution series
20. Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (Significant difference from control)
21. Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period.
22. Sample volume required:	20 L for effluents 40 L for receiving waters
23. Test acceptability criterion:	90% or greater survival in controls

TABLE 15. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR MYSID, *MYSIDOPSIS BAHIA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through
2. Test duration:	24, 48, or 96 h
3. Temperature:[2]	20°C ± 1°C; or 25°C ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	250 mL (minimum)
8. Test solution volume:	200 mL (minimum)
9. Renewal of test solutions:	Minimum, after 48 h
10. Age of test organisms:	1-5 days; 24-h range in age
11. No. organisms per test chamber:	Minimum, 10 for effluent and receiving water tests
12. No. replicate chambers per concentration:	Minimum, 2 for effluent tests Minimum, 4 for receiving water tests
13. No. organisms per concentration:	Minimum, 20 for effluent tests Minimum, 40 for receiving water tests
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; feed 0.2 mL of concentrated suspension of *Artemia* nauplii ≤24-h old, daily (approximately 100 nauplii per mysid)

[1] *Homesimysis costata* (mysid) can be used with the test conditions in this table (except at a temperature of 12°C or 25°C, and a salinity of 32-34%, where it is the required test organism in discharge permits.

[2] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and salinity.

TABLE 15. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR MYSID, *MYSIDOPSIS BAHIA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	5-30‰ ± 10%; Uncontaminated source of seawater, deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, modified GP2, or equivalent) prepared with MILLI-Q®, or equivalent, deionized water (see Section 7, Dilution Water); or receiving water
18. Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19. Dilution series:	Effluents: ≥0.5 dilution series Receiving Waters: None, or ≥0.5 dilution series
20. Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (Significant difference from control)
21. Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period.
22. Sample volume required:	1 L for effluents 2 L for receiving waters
23. Test acceptability criterion:	90% or greater survival in controls

TABLE 16. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal, static-renewal, or flow-through
2. Test duration:	24, 48, 96 h
3. Temperature:[1]	20°C ± 1°C; or 25°C ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	250 mL (minimum)
8. Test solution volume:	200 mL (minimum)
9. Renewal of test solutions:	Minimum, after 48 h
10. Age of test organisms:	1-14 days; 24-h range in age
11. No. organisms per test chamber:	Minimum, 10 for effluent and receiving water tests
12. No. replicate chambers per concentration:	Minimum, 2 for effluent tests Minimum, 4 for receiving water tests
13. No. organisms per concentration:	Minimum, 20 for effluent tests Minimum, 40 for receiving water tests
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h
15. Test chamber cleaning:	Cleaning not required

[1]Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and salinity.

TABLE 16. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	5-32‰ ± 10%; Uncontaminated source of seawater, deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, modified GP2, or equivalent) prepared with MILLI-Q® or equivalent deionized water (see Section 7, Dilution Water); or receiving water
18. Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19. Dilution series:	Effluents: ≥0.5 dilution series Receiving Waters: None, or >0.5 dilution series
20. Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (Significant difference from control)
21. Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period.
22. Sample volume required:	1 L for effluents 2 L for receiving waters
23. Test acceptability criterion:	90% or greater survival in controls

TABLE 16. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SHEEPSHEAD MINNOW, CYPRINODON VARIEGATUS, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	5-32‰ ± 10‰; Uncontaminated source of seawater, deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, modified GP2, or equivalent) prepared with MILLI-Q® or equivalent deionized water (see Section 7, Dilution Water); or receiving water
18. Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19. Dilution series:	Effluents: ≥0.5 dilution series Receiving Waters: None, or ≥0.5 dilution series
20. Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (Significant difference from control)
21. Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period.
22. Sample volume required:	1 L for effluents 2 L for receiving waters
23. Test acceptability criterion:	90% or greater survival in controls

TABLE 17. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SILVERSIDE, *MENIDIA BERYLLINA*, *M. MENIDIA*, AND *M. PENINSULAE*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal, static-renewal, or flow-through
2. Test duration:	24, 48, or 96 h
3. Temperature:[1]	20°C ± 1°C; or 25°C ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	250 mL (minimum)
8. Test solution volume:	200 mL (minimum)
9. Renewal of test solutions:	Minimum, after 48 h
10. Age of test organisms:	9-14 days; 24-h range in age
11. No. organisms per test chamber:	Minimum, 10 for effluent and receiving water tests
12. No. replicate chambers per concentration:	Minimum, 2 for effluent tests Minimum, 4 for receiving water tests
13. No. organisms per concentration:	Minimum, 20 for effluent tests Minimum, 40 for receiving water tests
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h
15. Test chamber cleaning:	Cleaning not required

[1]Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and salinity.

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METHODS FOR MEASURING THE ACUTE TOXICITY OF EFFLUENTS AND RECEIVING WATERS TO FRESHWATER AND MARINE ORGANISMS

(Fourth Edition)

Edited by

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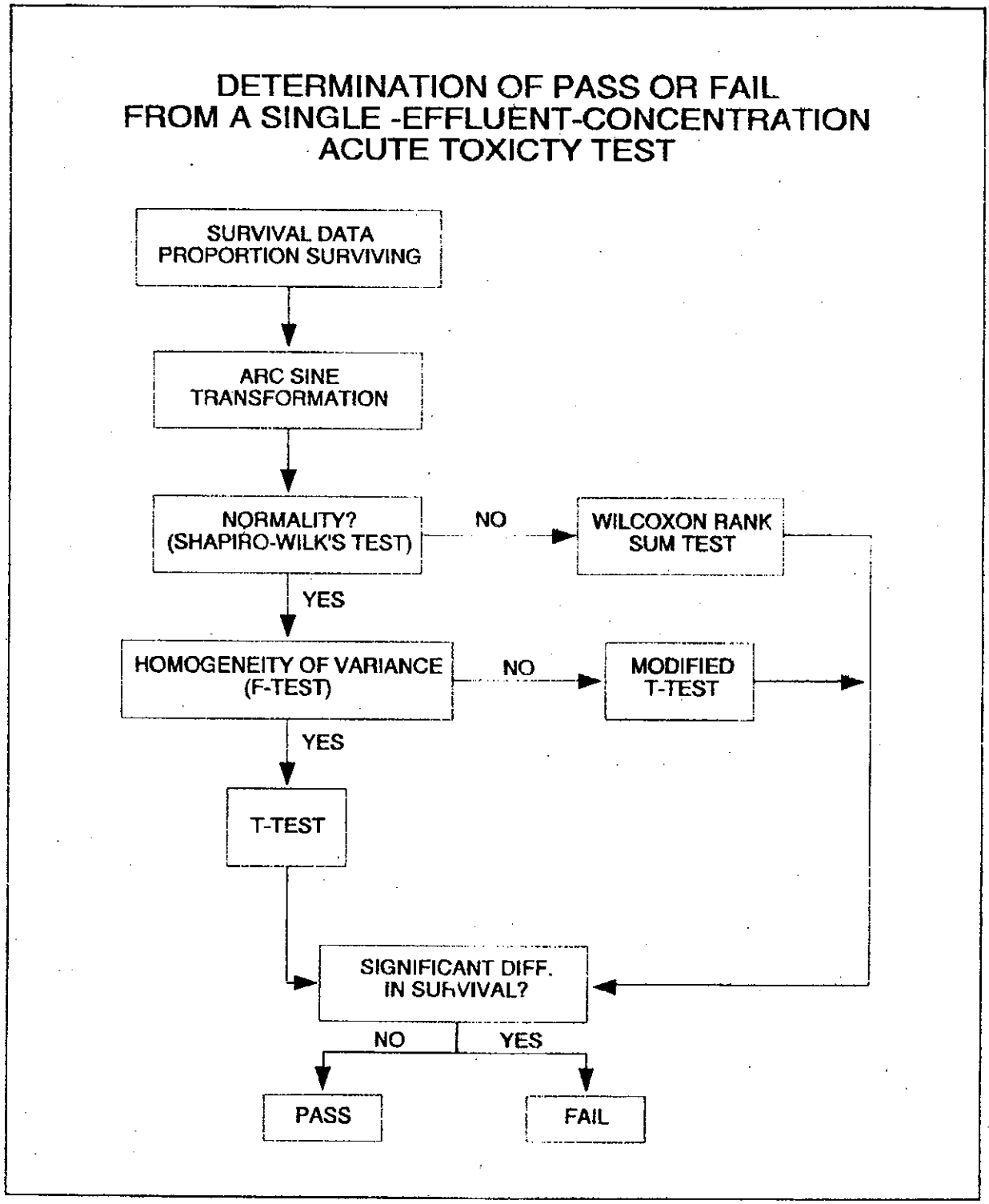

Figure 12. Flowchart for analysis of single-effluent-concentration test data.

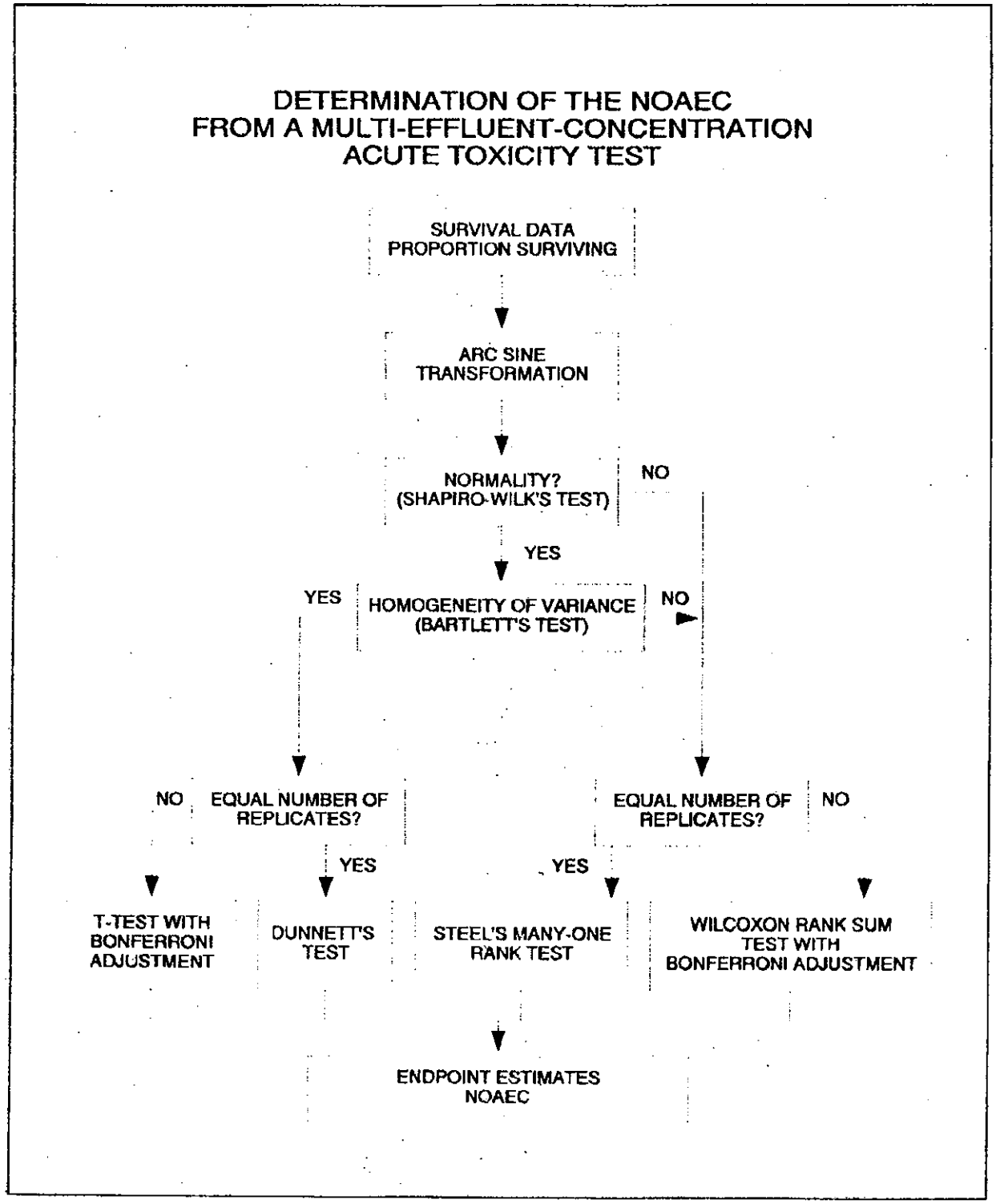

Figure 13. Flowchart for analysis of multi-effluent-concentration test data.

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

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 19, 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 20 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.

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

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 19. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST[1] (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 19. 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 20. 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

11.3.5.4.2 Calculate the following test statistic:

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

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 nonparametric Wilcoxon Rank Sum Test may be used to analyze the data. If less than four replicates were used, a nonparametric 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 survival in the control. For a test at the 5% level of significance, the critical rank sum can be found in Table 21.

TABLE 21. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST AT THE FIVE PERCENT SIGNIFICANCE LEVEL

NO. 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 22. The proportion surviving in each replicate is transformed by the arc sine square root transformation prior to statistical analysis of the data (Figure 12).

11.3.6.2 After the data have been transformed, test the assumption of normality with 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 23.

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

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

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

TABLE 22. DATA FROM AN ACUTE SINGLE-CONCENTRATION TOXICITY TEST WITH *CERIODAPHNIA*

	REPLICATE	PROPORTION SURVIVING	
		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 23. EXAMPLE OF SHAPIRO-WILK'S TEST: CENTERED OBSERVATIONS

TREATMENT	REPLICATE			
	A	B	C	D
Control	0.082	0.082	-0.081	-0.081
100% Effluent	0.081	-0.024	0.081	-0.140

11.3.6.2.3 Order the centered observations from smallest to largest. The ordered observations are listed in Table 24.

11.3.6.2.4 From Table 1, for n = 8 and k = n/2 = 4, obtain the coefficients $a_1, a_2, \ldots, a_k$. The a_i values are listed in Table 25.

TABLE 24. 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

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 25.

11.3.6.2.6 From Table 20, 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.

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

TABLE 25. 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	$X^{(5)} - X^{(4)}$

11.3.6.3.1 From Table 22, 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 $\bar{X}_1$, $\bar{X}_2$, S_1^2, and S_2^2 from Table 22. 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 with 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 26.

TABLE 26. 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 21, 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 27. A plot of the survival proportions is provided in Figure 14.

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 28.

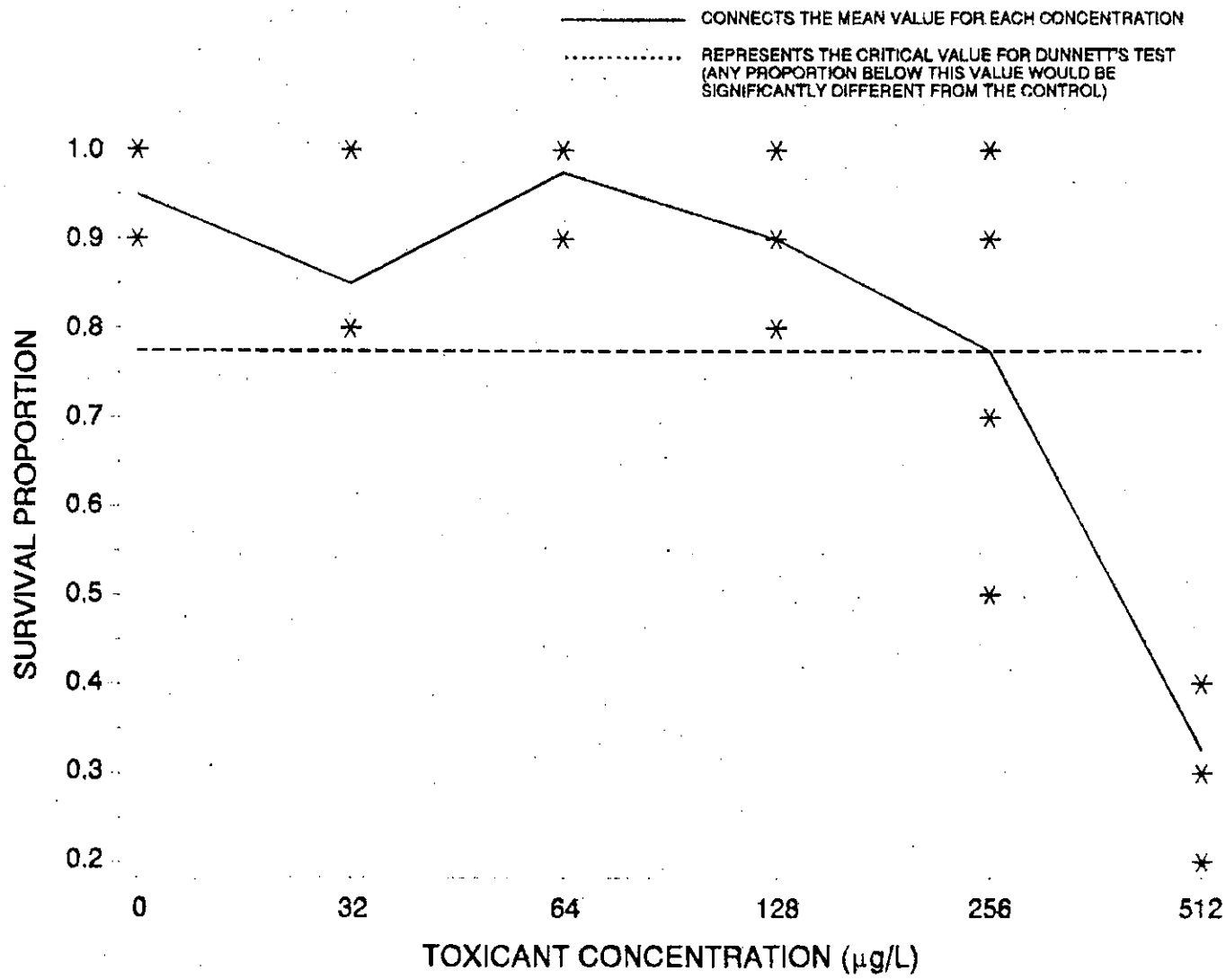

Figure 14. Plot of mean survival proportion data in Table 27.

TABLE 27. FATHEAD MINNOW SURVIVAL DATA

			TOXICANT CONCENTRATION (UG/L)				
	REPLICATE	CONTROL	32	64	128	256	512
RAW	A	1.0	0.8	0.9	0.9	0.7	0.4
	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 TRANS-FORMED	A	1.412	1.107	1.249	1.249	0.991	0.685
	B	1.412	1.107	1.412	1.249	1.249	0.580
	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 28. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		TOXICANT CONCENTRATION (UG/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 29.

TABLE 29. 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 17, 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 30.

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

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

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 30. For the data in this example,

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

TABLE 30. 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	$X^{(13)} - X^{(12)}$

7. The decision rule for this test is to compare W as calculated in #6 to a critical value found in Table 21. 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.

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:

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

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.

ln = $\log_e$

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 27) 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 31).

TABLE 31. ANOVA TABLE

SOURCE	DF	SUM OF SQUARES (SS)	MEAN SQUARE(MS) (SS/DF)
BETWEEN	P - I	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 $n_1 + n_2 \ldots + n_p$
n_i = number of observations in concentration i

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

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

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:

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

$$N = 24$$

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

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

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

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

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

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

$$SSW = SST - SSB = 2.000 - 1.574 = 0.4260$$
$$S_{B_2} = SSB/(p - 1) = 1.574/(6 - 1) = 0.3150$$
$$S_W = SSW/(N - p) = 0.426/(24 - 6) = 0.024$$

3. Summarize these calculations in the ANOVA table (Table 32).

TABLE 32. 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:

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

Where $\bar{Y}_i$ = mean proportion surviving for concentration i
$\bar{Y}_1$ = mean proportion surviving for the control
S_w = square root of the within mean square
n_1 = number of replicates for the control
n_i = number of replicates for concentration i.

5. Table 33 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 34. 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_6 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 33. 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.

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

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

	(One-tailed) α								
	α = .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
∞	1.64	1.92	2.06	2.16	2.23	2.29	2.34	2.33	2.42

TABLE 34. DUNNETT'S "T" VALUES (CONTINUED) (MILLER, 1981)

(One-tailed) α^*									
	$\alpha = 0.1$								
	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
∞	2.33	2.56	2.68	2.77	2.34	2.90	2.83	2.97	3.00

SECTION 12

REPORT PREPARATION

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

12.1 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

12.2 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

12.3 SOURCE OF EFFLUENT, RECEIVING WATER, AND DILUTION WATER

1. Effluent Samples
   a. Sampling point
   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
   b. Sample collection method
   c. Collection dates and times
   d. Streamflow at time of sampling and 7Q10
   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.)

12.4 **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)

12.5 **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)

12.6 **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

12.7 **RESULTS**

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

12.8 **CONCLUSIONS AND RECOMMENDATIONS**

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

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APPENDICES

APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.1. *CERIODAPHNIA DUBIA*

1. SYSTEMATICS

1.1 MORPHOLOGY AND TAXONOMY

1.1.1 *Ceriodaphnia* are closely related and morphologically similar to *Daphnia*, but are smaller and have a shorter generation time (USEPA, 1986). They are generally more rotund, lack the prominent rostral projection typical of *Daphnia*, and do not develop the dorsal helmets and long posterior spines often observed in *Daphnia*.

1.1.2 With *Ceriodaphnia dubia*, the female has a heavy, setulated pecten on the postabdominal claw (Figure 1A), and the male was long antennules (Figure 1C), in contrast to the closely related *C. reticulata*, where the female has heavy, triangular denticles in the pecten of the postabdominal claw (Figure 2D), and the male has very short antennules (Figure 2C). Some clones having intermediate characters may be hybrids or phenotypic variants of *C. dubia* (USEPA, 1986). Detailed descriptions of the males and females of both species and the variant were given by USEPA (1986).

1.1.3 Although males are very similar to females, they can be recognized by their rapid, erratic swimming habit, smaller size, denser coloration, extended antennules and claspers, and rostrum morphology.

2. ECOLOGY AND LIFE HISTORY

2.1 DISTRIBUTION

2.1.1 *C. dubia*, has been reported from littoral areas of lakes, ponds, and marshes throughout most of the world, but it is difficult to ascertain its true distribution because it has been reported in the literature under several other names (*C. affinis, C. quadrangula*, and *C. reticulata*. It has also been suggested that reports of *C. dubia* in New Zealand and parts of Asia may be yet another unnamed species (Berner, personal communication).

2.2 ECOLOGY

2.2.1 *Ceriodaphnia* ecology and life history are very similar to those of other daphnids. Specific information on the ecology and life history of *Ceriodaphnia dubia* is either not available or is widely scattered throughout the literature. However, it is known to be a pond and lake dwelling species that is usually common among the vegetation in littoral areas (Fairchild, 1981). In the Lake of Velence, Hungary, *C. dubia* was most common in regions where "grey" and "dark brown" waters merged (Pal, 1980). In Par Pond (Savannah River Plant, Aiken, SC) the *Ceriodaphnia* were much more abundant in the heated water (effluent from the nuclear reactor) than in the ambient area

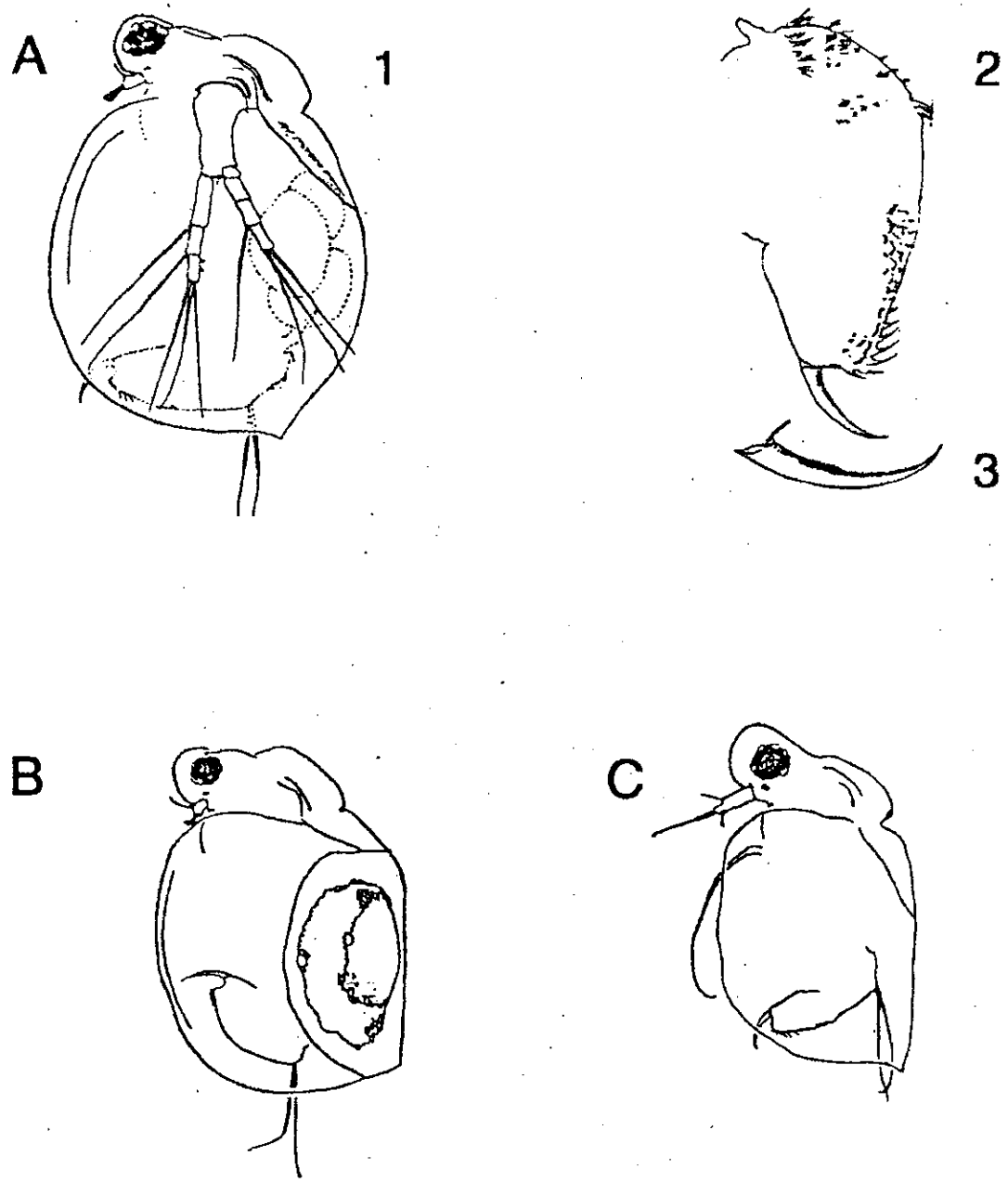


Figure 1. *Ceriodaphnia dubia*. A. (1) parthenogenetic female, (2) postabdomen, and (3) claw; B. ephippial female; C. male. (From USEPA, 1986).

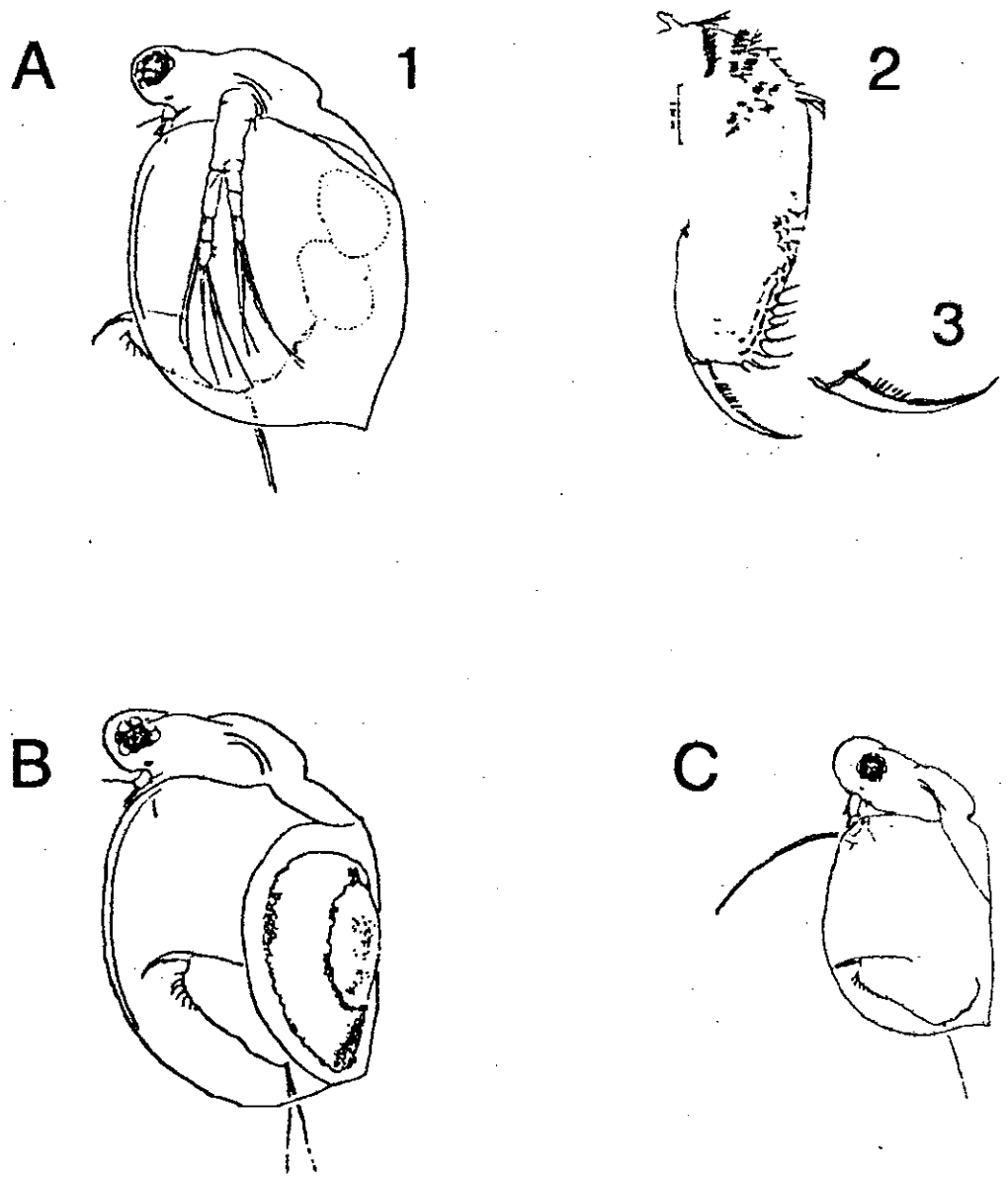


Figure 2. *Ceriodaphnia reticulata*. A. (1) parthenogenetic female, (2) postabdomen, (3) and claw; B. ephippial female; C. male. (From USEPA, 1986).

(Vigerstad and Tilly, 1977), and in a reservoir in Russia, animals from the heated water were larger and heavier than those living under normal water temperatures (Kititsyna and Sergeeva, 1976). In Iran they are common in warmer, montane, oligotrophic lakes (Smagowicz, 1976).

2.2.2 In Lake Kinneret, Israel, *Ceriodaphnia reticulata* are abundant only between March and June, with a peak in May when the temperature ranges between 20 and 22°C. When summer temperatures reached 27-28°C, the *Ceriodaphnia* were reduced in size and egg production became significantly less, leading to a progressive decline of the population (Gophen, 1976). In Lake Parvin, France, the period of development was from June to September (Devaux, 1980).

2.2.3 *Ceriodaphnia* typically swim with an erratic, jerking motion for a period of time, and hang motionless in the water between swimming bouts. This swimming behavior results in a mean speed of 1.5-2.5 mm/s. When approached by a predator, however, it flees by swimming away quickly along a straight path (Wong, 1981).

2.2.4 During most of the year, populations of *Ceriodaphnia* consist almost entirely of females; the males appearing principally in autumn. Production of males appears to be induced primarily by low water temperatures, high population densities, and/or a decrease in available food. As far as is presently known, *C. dubia* reproduce only by cyclic parthenogenesis in which the males contribute to the genetic makeup of the young during the sexual stage of reproduction.

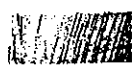
2.2.5 The females tend to aggregate during sexual reproductive activity, when ephippia are produced (Brandl and Fernando, 1971). Ephippia are embryos encased in a tough covering, and are resistent to drying. They can be stored for long periods and shipped through the mail in envelopes, like seeds. When placed in water at the proper temperature, ephippia hatch in a few days producing a new parthenogenetic population.

2.2.6 *Ceriodaphnia* have many predators, including fish, the mysid *Mysis relicta*, *Chaoborus* larvae, and copepods. As with *Daphnia*, it also reacts to intense predation with defensive strategies. *Ceriodaphnia reticulata* (possibly *C.dubia*) in a Minnesota lake, reacted to the copepod, *Cyclops vernalis*, by producing large offspring and growing to a large size at the expense of early reproduction (Lynch, 1979). They reacted to fish predators by producing smaller offspring in larger numbers.

2.3 FOOD AND FEEDING

2.3.1 Cladocera are polyphagous feeders and find their food in the seston. Daphnids, including the *Ceriodaphnia*, are classified as fine mesh filter feeders by Geller and Mueller (1981). These fine mesh filter feeders are most abundant in eutrophic lakes during summer phytoplankton blooms when suspended bacteria are available as food only for filter-feeding species with fine mesh.

2.3.2 Lynch (1978) examined the gut contents of *Ceriodaphnia reticulata* (possibly *C. dubia*) from a Minnesota pond and found bacteria, detritus and partially digested algae. In this pond, *Ceriodaphnia* and *Daphnia pulex* shared



the same resource base and had very similar diets, but the *Ceriodaphnia* fed more intensively on diatoms. The *Ceriodaphnia* were considered to be less sensitive to low food levels than *Daphnia*, because of their high rate of population growth during periods of low food levels in late summer.

2.4 LIFE CYCLE

2.4.1 Four distinct periods may be recognized in the life cycle of *Ceriodaphnia*: (1) egg, (2) juvenile, (3) adolescent, and (4) adult. The life span of *Ceriodaphnia*, from the release of the egg into the brood chamber until the death of the adult, is highly variable depending on the temperature and other environmental conditions. Generally the life span increases as temperature decreases, due to lowered metabolic activity. For example, the average life span of *Ceriodaphnia dubia* is about 30 days at 25°C, and 50 days at 20°C. One female was reported to have lived 125 days and produced 29 broods at 20°C (Cowgill et al., 1985).

2.4.2 Typically, a clutch of 4 to 10 eggs is released into the brood chamber, but clutches with as many as 20 eggs are common. The eggs hatch in the brood chamber and the juveniles, which are already similar in form to the adults, are released in approximately 38 h, when the female molts (casts off her exoskeleton or carapace). The total number of young produced per female varies with temperature and other environmental conditions. The most young are produced in the range of 18-25°C (124 young per female in a 28-day life span at 24°C) (113 young per female in a 77-day life span at 18°C) but production falls off sharply below 18°C (13 young per female in a 24-day life span at 12°C) (McNaught and Mount, 1985).

2.4.3 The time required to reach maturity (produce their first offspring) in *C.dubia* varies from three to five days and appears to be dependent on body size and environmental conditions. A study of the growth and development of parthenogenetic eggs by Shuba and Costa (1972) revealed that at 24°C the embryos matured to free-swimming juveniles in approximately 38 h. The eggs that did not develop fully usually were aborted after 12 hours.

2.4.4 The growth rate of the organism is greatest during its juvenile stages (early instars), and the body size may double during each of these stages. Each instar stage is terminated by a molt. Growth occurs immediately after each molt while the new carapace is still elastic.

2.4.5 Following the juvenile stages, the adolescent period is very short, and consists of a single instar. It is during the adolescent instar that the first clutch of eggs reaches full development in the ovary. Generally, eggs are deposited in the brood chamber within minutes after molting, and the young which develop are released just before the next molt.

2.4.6 In general, the duration of instars increases with age, but also depends on environmental conditions. A given instar usually lasts approximately 24 h under favorable conditions. However, when conditions are unfavorable, it may last as long as a week. Four events take place in a matter of a few minutes at the end of each adult instar: (1) release of young from the brood chamber to the outside, (2) molting, (3) increase in size, and

(4) release of a new clutch of eggs into the brood chamber. The number of young per brood is highly variable, depending primarily on food availability and environmental conditions. *C. dubia* may produce as many as 25 young in a single brood, but more commonly the number is six to ten. The number of young released during the adult instars reaches a maximum at about the fourth instar, after which there is a gradual decrease.

3. CULTURING METHODS

3.1 *Ceriodaphnia* are available from commercial biological supply houses. Guidance on the source of culture animals to be used by a permittee for self-monitoring effluent toxicity tests should be obtained from the permitting authority. Only a small number of organisms (20-30) are needed to start a culture. Before test organisms are taken from a culture, the culture should be maintained for at least two generations using the same food, water, and temperature as will be used in the toxicity tests.

3.2 Cultures of test organisms should be started at least three weeks before the brood animals are needed, to ensure an adequate supply of neonates for the test. Only a few individuals are needed to start a culture because of their prolific reproduction.

3.3 Starter animals may be obtained from an outside source by shipping in polyethylene bottles. Approximately 20-30 animals and 3 mL of food (see below) are placed in a 1-L bottle filled full with culture water. Animals received from an outside source should be transferred to new culture media gradually over a period of 1-2 days to avoid mass mortality.

3.4 It is best to start the cultures with one animal, which is sacrificed after producing young, embedded, and retained as a permanent microscope slide mount to facilitate identification and permit future reference. The species identification of the stock culture should be verified by a taxonomic authority. The following procedure is recommended for making slide mounts of *Ceriodaphnia* (Beckett and Lewis, 1982):

1. Pipet the animal onto a watch glass.
2. Reduce the water volume by withdrawing excess water with the pipet.
3. Add a few drops of carbonated water (club soda or seltzer water) or 70% ethanol to relax the specimen so that the post-abdomen is extended. (Optional: with practice, extension of the postabdomen may be accomplished by putting pressure on the cover slip).
4. Place a small amount (one to three drops) of mounting medium on a glass microscope slide. The recommended mounting medium is CMCP-9/9AF Medium, prepared by mixing two parts of CMCP-9 with one part of CMCP-9AF. For more viscosity and faster drying, CMC-10 stained with acid fuchsin may be used. CMCP-9 and 9AF are available from Polysciences, Inc., Paul Valley Industrial Park, Warrington, PA 18976 (215-343-6484).
5. Using a forceps or a pipet, transfer the animal to the drop of mounting medium on the microscope slide.

6. Cover with a cover slip and exert minimum pressure to remove any air bubbles trapped under the cover slip. Slightly more pressure will extend the postabdomen.
7. Allow mounting medium to dry.
8. Make slide permanent by placing CMC-10 around the edges of the coverslip.
9. Identify to species (see Pennak, 1989, and USEPA, 1986).
10. Label with waterproof ink or diamond pencil.
11. Store for permanent record.

3.5 CULTURE MEDIA

3.5.1 Although *Ceriodaphnia* stock cultures can be successfully maintained in some tap waters, well waters, and surface waters, use of synthetic water as the culture medium is recommended because (1) it is easily prepared, (2) it is of known quality, (3) it yields reproducible results, and (4) allows adequate growth and reproduction. Culturing may be successfully done in hard, moderately hard or soft reconstituted water, depending on the hardness of the water in which the test will be conducted. The quality of the dilution water is extremely important in *Ceriodaphnia* culture. The use of MILLIPORE MILLI-Q® or SUPER-Q®, or equivalent, to prepare reconstituted water is highly recommended. The use of diluted mineral water (DMW) for culturing and testing is widespread due to the ease of preparation.

3.5.2 The chemicals used and instructions for preparation of reconstituted water are given in Section 7, Dilution Water. The compounds are dissolved in distilled or deionized water and the media are vigorously aerated for several hours before using. The initial pH of the media is between 7.0 and 8.0, but it will rise as much as 0.5 unit after the test is underway.

3.6 MASS CULTURE

3.6.1 Mass cultures are used only as a "backup" reservoir of organisms. Neonates from mass cultures are not to be used directly in toxicity tests.

3.6.2 One-liter or 2L glass beakers, crystallization dishes, "battery jars," or aquaria may be used as culture vessels. Vessels are commonly filled to three-fourths capacity. Cultures are fed daily. Four or more cultures are maintained in separate vessels and with overlapping ages to serve as back-up in case one culture is lost due to accident or other unanticipated problems, such as low DO concentrations or poor quality of food or laboratory water.

3.6.3 Mass cultures which will serve as a source of brood organisms for individual culture should be maintained in good condition by frequent renewal of the medium and brood organisms. Cultures are started by adding 40-50 neonates per liter of medium. The stocked organisms should be transferred to new culture medium at least twice a week for two weeks. After two weeks, the culture is discarded and re-started with neonates in fresh medium. Using this schedule, 1-L cultures will produce 500 to 1000 neonate *Ceriodaphnia* each week.

3.6.6 Reserve cultures also may be maintained in large (80-L) aquaria or other large tanks.

3.7 INDIVIDUAL CULTURE

3.7.1 Individual cultures are used as the immediate source of neonates for toxicity tests.

3.7.2 Individual organisms are cultured in 15 mL of culture medium in 30-mL (1 oz) plastic cups or 30-mL glass beakers. One neonate is placed in each cup. It is convenient to place the cups in the same type of board used for toxicity tests (see Figure 1).

3.7.3 Organisms are fed daily and are transferred to fresh medium a minimum of three times a week, typically on Monday, Wednesday, and Friday. On the transfer days, food is added to the new medium immediately before or after the organisms are transferred.

3.7.4 To provide cultures of overlapping ages, new boards are started weekly, using neonates from adults which produce at least eight young in their third or fourth brood. These adults can be used as sources of neonates until 14 days of age. A minimum of two boards are maintained concurrently to provide backup supplies of organisms in case of problems.

3.7.5 Cultures which are properly maintained should produce at least 20 young per adult in three broods (seven days or less at 25°C). Typically, 60 adult females (one board) will produce more than the minimum number of neonates (120) required for two tests.

3.7.6 Records should be maintained on the survival of brood organisms and number of offspring at each renewal. Greater than 20% mortality of adults or less than an average of 20 young per adult on a board at 25°C during a one-week period would indicate problems, such as poor quality of culture media or food. Organisms on that board should not be used as a source of test organisms.

3.8 CULTURE MEDIUM

3.8.1 Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW is recommended as a standard culture medium (see Section 7, Dilution Water).

3.9 CULTURE CONDITIONS

3.9.1 *Ceriodaphnia* should be cultured at the temperature at which they will be used in the toxicity tests (20°C or 25°C ± 2°C).

3.9.2 Day/night cycles prevailing in most laboratories will provide adequate illumination for normal growth and reproduction. A 16-h/8-h day/night cycle is recommended.

3.9.3 Clear, double-strength safety glass or 6 mm plastic panels are placed on the culture vessels to exclude dust and dirt, and reduce evaporation.

3.9.4 The organisms are delicate and should be handled as carefully and as little as possible so that they are not unnecessarily stressed. They are transferred with a pipet of approximately 2-mm bore, taking care to release the animals under the surface of the water. Any organism that is injured during handling should be discarded.

3.10 FOOD PREPARATION AND FEEDING

3.10.1 Feeding the proper amount of the right food is extremely important in Ceriodaphnia culturing. The key is to provide sufficient nutrition to support normal reproduction without adding excess food which may reduce the toxicity of the test solutions, clog the animal's filtering apparatus, or greatly decrease the DO concentration and increase mortality. A combination of Yeast, CEROPHYLL®, and Trout chow (YCT) or flake food, along with the unicellular green alga, *Selenastrum capricornutum*, will provide suitable nutrition if fed daily.

3.10.2 The YCT and algae are prepared as follows:

3.10.2.1 Digested trout chow (or flake food):

1. Preparation of trout chow requires one week. Use starter or No. 1 pellets prepared according to current U.S. Fish and Wildlife Service specifications, or flake food. Suppliers of trout chow include Zeigler Bros., Inc., P.O. Box 95, Gardners, PA 17324 (717-780-9009); Glencoe Mills, 1011 Elliott, Glencoe, MN 55336 (612-864-3181); and Murray Elevators, 118 West 4800 South, Murray, UT 84107 (800-521-9092).

2. Add 5.0 g of trout chow pellets or flake food to 1 L of MILLI-Q® water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation is replaced during digestion. Because of the offensive odor usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.

3. At the end of digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (i.e., NITEX® 110 mesh). Combine with equal volumes of supernatant from CEROPHYLL® and yeast preparations (below). The supernatant can be used fresh, or frozen until use. Discard the sediment.

3.10.2.2 Yeast:

1. Add 5.0 g of dry yeast, such as FLEISCHMANN'S® to 1 L of MILLI-Q® water.
2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.
3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow (above) and CEROPHYLL® preparations (below). Discard excess material.

3.10.2.3 CEROPHYLL® (Dried, Powdered, Cereal Leaves):

1. Place 5.0 g of dried, powdered, cereal leaves in a blender. (Available as "CEREAL LEAVES," from Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 (800-325-3010); or as CEROPHYLL®, from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY, 14692-9012 (716-359-2502). Dried, powdered, alfalfa leaves obtained from health food stores have been found to be a satisfactory substitute for cereal leaves.
2. Add 1 L of MILLI-Q® water.
3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.
4. If a blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow to settle for 1 h. Decant the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard excess material.

3.10.2.4 Combined YCT Food:

1. Mix equal (approximately 300 mL) volumes of the three foods as described above.
2. Place aliquots of the mixture in small (50-mL to 100-mL) screw-cap plastic bottles and freeze until needed.
3. Freshly prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings, and is used for a maximum of two weeks.
4. It is advisable to measure the dry weight of solids (dry 24 h at 105°C) in each batch of YCT before use. The food should contain 1.7-1.9 g solids/L. Cultures or test solutions should contain 12-13 mg solids/L.

3.10.3 Algal (*Selenastrum*) Food

3.10.3.1 Algal Culture Medium

1. Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.
2. Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q® water. Mix well after the addition of each solution. Dilute to 1 L and mix well. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.
3. Immediately filter the medium through a 0.45 μm pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.
4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels.
5. Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

TABLE I. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 ML MILLI-Q® WATER
1. MACRONUTRIENTS		
A.	$MgCl_2.6H_2O$	6.08 g
	$CaCl_2.2H_2O$	2.20 g
	$NaNO_3$	12.75 g
B.	$MgSO_4.7H_2O$	7.35 g
C.	K_2HPO_4	0.522 g
D.	$NaHCO_3$	7.50 g
2. MICRONUTRIENTS		
	H_3BO_3	92.8 mg
	$MnCl_2.4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg[a]
	$FeCl_3.6H_2O$	79.9 mg
	$CoCl_2.6H_2O$	0.714 mg[b]
	$Na_2MoO_4.2H_2O$	3.63 mg[c]
	$CuCl_2.2H_2O$	0.006 mg[d]
	$Na_2EDTA.2H_2O$	150.0 mg
	Na_2SeO_4	1.196 mg[e]

[a] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.

[b] $CoCl_2.6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to stock #1.

[c] $Na_2MoO_4.2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to stock #1.

[d] $CuCl_2.2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock #1.

[e] Na_2SeO_4 - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.

TABLE 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

Macronutrient	Concentration (mg/L)	Element	Concentration (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2.6H_2O$	12.2	Mg	2.90
$CaCl_2.2H_2O$	4.41	Ca	1.20
$MgSO_4.7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

Micronutrient	Concentration ($\mu g/L$)	Element	Concentration ($\mu g/L$)
H_3BO_3	185	B	32.5
$MnCl_2.4H_2O$	416	Mn	115
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2.6H_2O$	1.43	Co	0.354
$CuCl_2.2H_2O$	0.012	Cu	0.004
$Na_2MoO_4.2H_2O$	7.26	Mo	2.88
$FeCl_3.6H_2O$	160	Fe	33.1
$Na_2EDTA.2H_2O$	300	--	----
Na_2SeO_4	2.39	Se	1.00

3.10.3.2 Algal Cultures

3.10.3.2.1 Two types of algal cultures are maintained: (1) stock cultures, and, (2) "food" cultures.

3.10.3.2.2 Establishing and Maintaining Stock Cultures of Algae

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring one milliliter to each of several 250-mL culture flasks containing 100 mL algal culture medium (prepared as described above). The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.
2. The stock cultures are used as a source of algae to initiate "food" cultures for *Ceriodaphnia* toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal food required for the *Ceriodaphnia* cultures and tests. Stock culture volume may be rapidly "scaled up" to several liters, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.
3. Culture temperature is not critical. Stock cultures may be maintained in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).
4. Cultures are mixed twice daily by hand or stirred continuously.
5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three milliliters of 7-day old algal stock culture, containing approximately 1.5 X 10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 10,000-30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.
6. Stock cultures should be examined microscopically weekly, at transfer, for microbial contamination. Reserve quantities of culture organisms can be maintained for 6-12 months if stored in the dark at 4°C. It is advisable to prepare new stock cultures from "starter" cultures obtained from established outside sources of organisms every four to six months.

3.10.3.2.3 Establishing and Maintaining "Food" Cultures of Algae

1. "Food" cultures are started seven days prior to use for *Ceriodaphnia* cultures and tests. Approximately 20 mL of 7-day-old algal stock culture (described in the previous paragraph), containing 1.5 X 10^6 cells/mL, are added to each liter of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of approximately 30,000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are terminated in 7-10 days. A one-month supply of algal food can be grown at one time, and the excess stored in the refrigerator.

2. Food cultures may be maintained at 25°C in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).
3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) or in a moderately aerated separatory funnel, or are mixed twice daily by hand. If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees. Caution should be exercised to prevent the culture temperature from rising more than 2-3°C.

3.10.3.3 Preparing Algal Concentrate for Use as *Ceriodaphnia* Food

1. An algal concentrate containing 3.0 to 3.5 X 10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for approximately two-to-three weeks and siphoning off the supernatant.
2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer, and used to determine the concentration required to achieve a final cell count of 3.0 to 3.5 X 10^7/mL.
3. Assuming a cell density of approximately 1.5 X 10^6 cells/mL in the algal food cultures at 7 days, and 100% recovery in the concentration process, a 3-L, 7-10 day culture will provide 4.5 X 10^8 algal cells. This number of cells would provide approximately 150 mL of algal cell concentrate (1500 feedings at 0.1 mL/feeding) for use as food. This would be enough algal food for four *Ceriodaphnia* tests.
4. Algal concentrate may be stored in the refrigerator for one month.

3.11 FEEDING

3.11.1 Cultures should be fed daily to maintain the organisms in optimum condition so as to provide maximum reproduction. Stock cultures which are stressed because they are not adequately fed may produce low numbers of young, large number of males, and ephippial females. Also, their offspring may produce few young when used in toxicity tests.

1. If YCT is frozen, remove a bottle of food from the freezer 1 h before feeding time, and allow to thaw.
2. Mass cultures are fed daily at the rate of 7 mL YCT and 7 mL algae concentrate/L culture.
3. Individual cultures are fed at the rate of 0.1 mL YCT and 0.1 mL algae concentrate per 15 mL culture.
4. YCT and algal concentrate should be thoroughly mixed by shaking before dispensing.
5. Return unused YCT food mixture and algae concentrate to the refrigerator. Do not re-freeze YCT. Discard unused portion after one week.

3.12 FOOD QUALITY

3.12.1 The quality of food prepared with newly acquired supplies of yeast, trout chow, dried cereal leaves, or algae, should be determined in side-by-side

comparisons of *Ceriodaphnia* survival and reproduction, using the new food and food of known, acceptable quality, over a seven-day period in control medium.

4. TEST ORGANISMS

4.1 Neonates, or first instar *Ceriodaphnia* less than 24 hours old, taken from the 3rd or 4th brood, are used in toxicity tests. To obtain the necessary number of young for an acute toxicity test, it is recommended that the animals be cultured in individual 30 mL beakers or plastic cups for seven days prior to the beginning of the test. Neonates are used from broods of at least eight young. Fifty adults in individual cultures will usually supply enough neonates for one toxicity test.

4.2 Use a disposable, widemouth pipette to transfer *Ceriodaphnia*. The diameter of the opening should be approximately 4 mm. The tip of the pipette should be kept under the surface of the water when the *Ceriodaphnia* are released to prevent air from being trapped under the carapace. Liquid containing adult *Ceriodaphnia* can be poured from one container to another without risk of injuring the animals.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.2. *DAPHNIA (D. MAGNA* AND *D. PULEX)*

1. SYSTEMATICS

1.1 MORPHOLOGY AND TAXONOMY

1.1.1 The generalized anatomy of a parthenogenetic female is shown in Figure 1. *Daphnia pulex* is an extremely variable species consisting of several reproductively isolated clonal groups and is often not distinguishable from other species (such as *D. obtusa*) that have large teeth on the middle pecten of the postabdomenal claw (Figure 2C) (Lynch, 1985; Dodson, 1981). Probably the most distinctive feature of the parthenogenetic female *D. pulex* is the long second abdominal process of the abreptor (postabdomen) that extends beyond the base of the anal setae (Figure 2A).

1.1.2 *D. pulex* is a wide ranging species that shows little variation throughout its range. Two of its most distinctive characteristics are the deeply sinuate posterior margin of the abreptor (Figures 3A and 3D) and the ridges on the head which run parallel to the mid-dorsal line (Figure 3B).

1.1.3 *D. pulex* is much smaller than *D. magna*, attaining a length of up to 3.5 mm compared to 5.0 or 6.0 mm for *D. magna*. Although the two species can often be separated by size, they can be differentiated with certainty only by examining the postabdominal claws for size and number of spines using a compound microscope. *D. pulex* has 5-7 stout teeth on the middle pecten (Figure 2C) while *D. magna* has a uniform row of 20 or more small teeth (Figure 3E). Another characteristic for separating the neonates of the two species is the location of the nuchal organ which is higher up on the posterior margin of the head in *D. magna* than in *D. pulex* (Schwartz and Hebert, 1984). For a more complete taxonomic discussion of the two species see Brooks (1957).

2. DISTRIBUTION

2.1 *D. magna* has a worldwide distribution in the northern hemisphere. In North America it appears to be absent from the eastern United States (except for Northern New England) and Alaska (Figure 4). *D. pulex* occurs over most of North America except the tropics and high arctic (Figure 5), and probably occurs in Europe and South America as well. Both species often occur in the same pools but *D. pulex* usually out-competes *D. magna* in mixed populations and takes over as the sole inhabitant by summer's end (Modlin, 1982; Lynch, 1983).

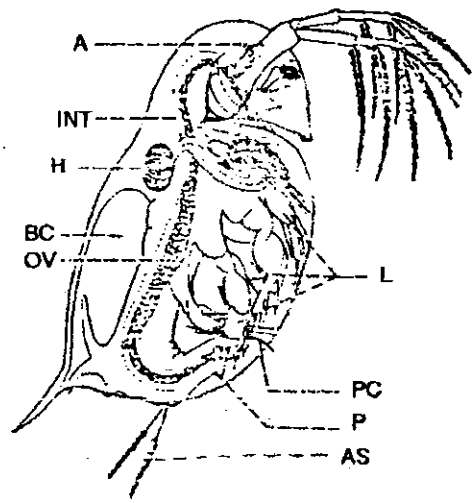

Figure 1. Generalized anatomy of a female *Daphnia*, X70; A, antenna; AS, anal setae; BC, brood chamber; H, heart; INT, intestine; L, legs; OV, ovary; P, postabdomen; PC, postabdominal claw. (From Pennak, 1989).

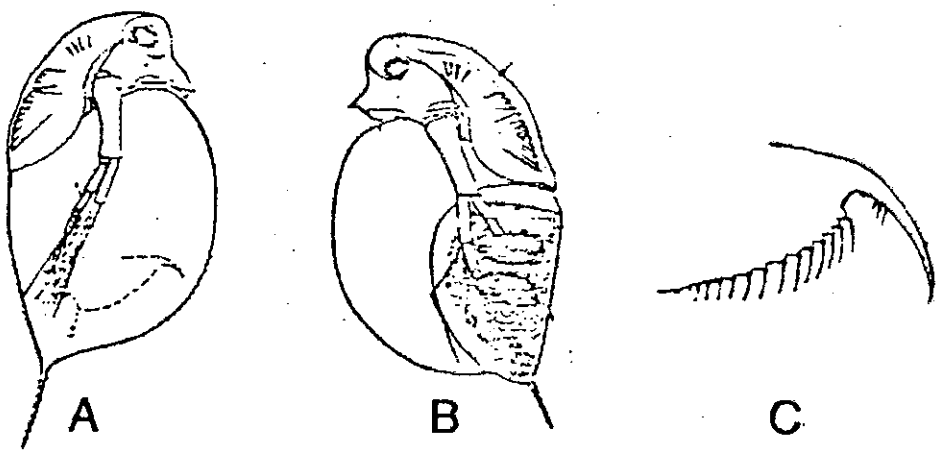

Figure 2. Female *Daphnia pulex*. A, lateral aspect (note smoothly rounded posterior margin of postabdomen); B, ephippial female; C, postabdomen showing large spines on the claw. (From Brooks, 1957).

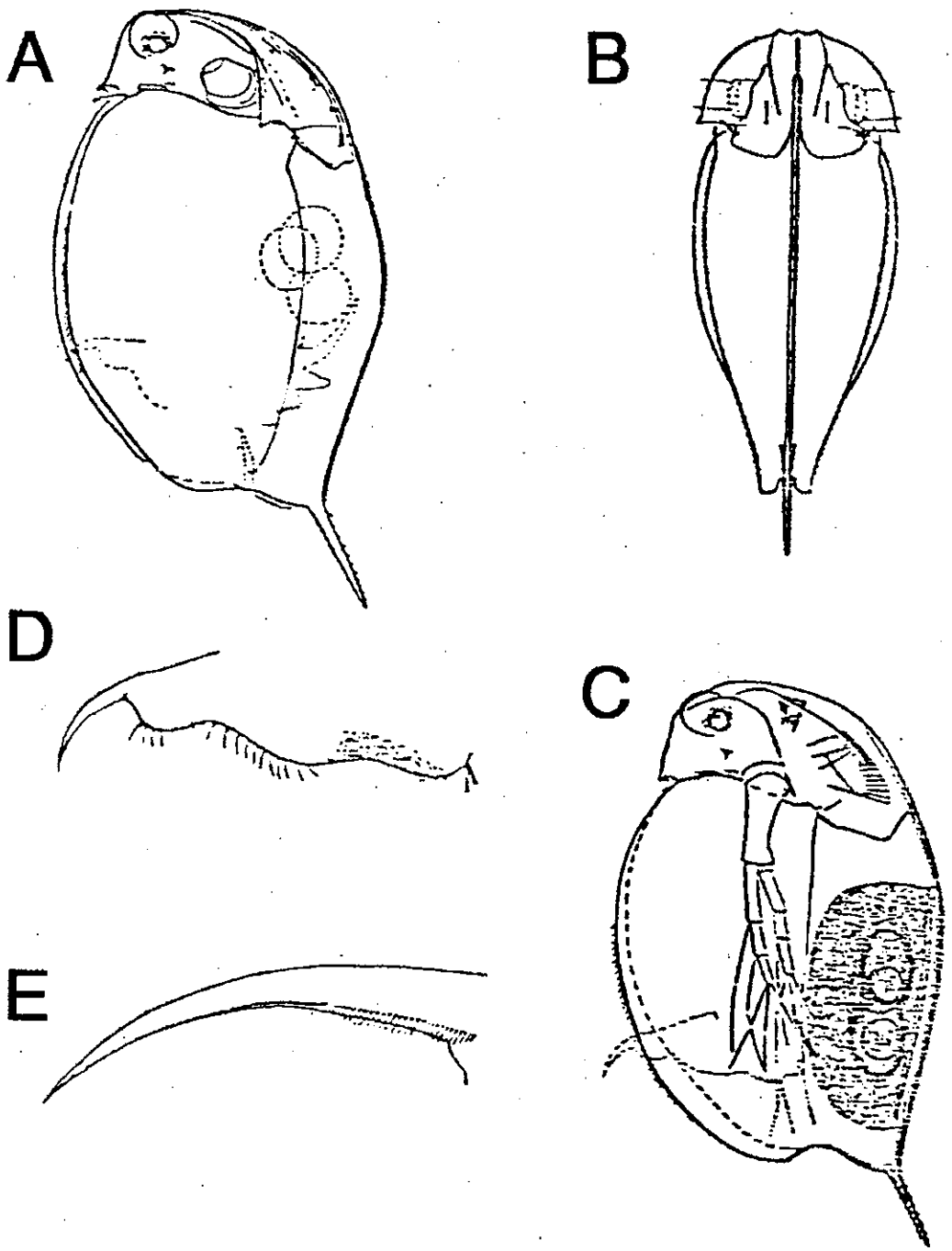

Figure 3. Female *D. magna*. A. Lateral aspect; B. dorsal aspect; C. ephippial female; D. postabdomen showing sinuate posterior margin; E. postabdominal claw. (From Brooks, 1957).

Figure 4. Map showing the North American distribution of *D. magna*.

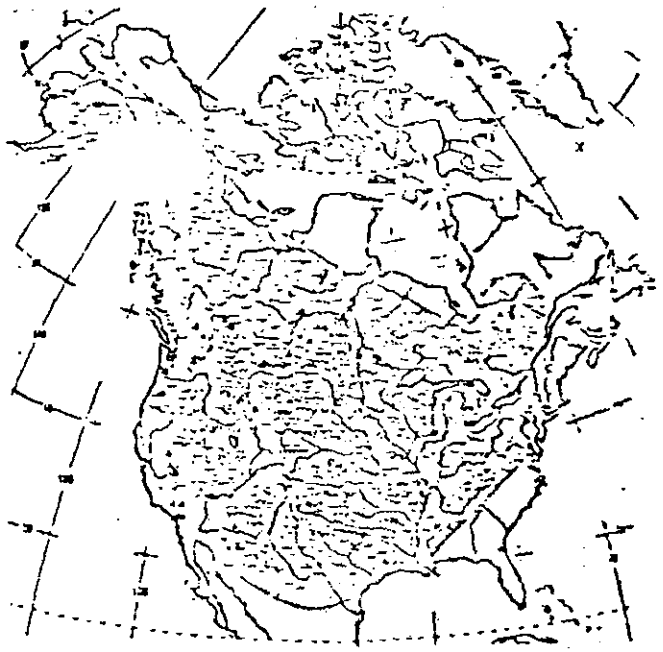

Figure 5. Map showing the North American distribution of *D. pulex*.

3. ECOLOGY AND LIFE HISTORY

3.1 GENERAL ECOLOGY

3.1.1 *D. magna* is principally a lake dweller and is restricted to waters in northern and western North America exceeding a hardness of 150 mg/L (as $CaCO_3$) (Pennak, 1989). In the Netherlands, *D. magna* are found in shallow ponds with muddy bottoms rich in organic matter and with low oxygen demand (3 to 4 mg/L). *D. pulex* is principally a pond dweller where the oxygen content is higher, but is also found in lakes. It is generally considered a clean water species being dominant in nature during periods of low turbidity. However, Scholtz, et al. (1988) found that high turbidity had little effect on survival and reproduction in laboratory studies.

3.1.2 *Daphnia* populations are generally sparse in winter and early spring, but as water temperatures reach 6°C to 12°C, they increase in abundance and subsequently may reach population densities as high as 200 to 500 individuals/L (Pennak, 1989). Populations in ponds decline to very low numbers during the summer months. In autumn there may be a second population pulse, followed by a decline to winter lows.

3.1.3 During most of the year, populations of *Daphnia* consist almost entirely of females, the males being abundant only in spring or autumn when up to 56% of the offspring of *D. magna* may be males (Barker and Hebert, 1986). Males are distinguished from females by their smaller size, larger antennules, modified postabdomen, and first legs, which are armed with a stout hook used in clasping. Production of males appears to be induced principally by low temperatures or high densities and subsequent accumulation of excretory products, and/or a decrease in available food. These conditions may induce the appearance of sexual (resting) eggs (embryos) in cases called ephippia (Figures 2B and 3C), which are cast off during the next molt. It appears that the shift toward male and sexual egg production is related to the metabolic rate of the parent. Any factor which tends to lower metabolism may be responsible. Ruvinsky et al. (1978) suggested that the genome of the animal has two developmental programs based on identical sets of chromosomes. The female program consistently functions under a wide range of conditions, whereas the male program is turned on by specific ecological stimuli. The eggs from which the males and females develop have identical chromosome sets. Sex determination is based on changes in chromatin structure when the mother receives a specific signal that sexual reproduction is needed for adaptation to extreme conditions.

3.1.4 *D. magna* reproduce only by cyclic parthenogenesis in which males contribute to the genetic makeup of the young during the sexual stage of reproduction, whereas *D. pulex* may reproduce either by cyclic or obligate parthenogenesis in which the zygotes develop within the ephippium by ameoitic parthenogenesis with no genetic contribution from the males. Thus, the ephippial and live-born offspring are genetically identical to their mothers. Both forms may be present in the same population resulting in cyclic populations exhibiting considerable genetic variation early in the year and an obligate population with a low range of genotypic values. After 25 generations of asexual reproduction the variation in the cyclic

parthenogenesis group becomes about the same as that in the obligate group (Lynch, 1984). These populations exhibiting a low range of genotypic values are much more vulnerable to perturbations such as nutrient introduction or toxic discharges. The clonal makeup of a *Daphnia* population is effected by food, oxygen, temperature and predation (Weider, 1985; Brookfield, 1984).

3.1.5 Ephippia are small and lightweight and can be dried and stored for long periods making them easy to transport. They may be shipped in envelopes like seeds. Upon arrival at the new location the ephippia can be hatched in a few days when placed in water at the proper temperature (Schwartz and Hebert, 1987).

3.1.6 *Daphnia* are preyed upon by many predators and have developed behavioral and morphological antipredator defenses to make themselves more difficult to catch and consume. Dodson (1988) showed that *D. pulex* responded to a possible chemical stimuli released by the predator which resulted in the daphnids retreating from the vicinity of the predators. Certain clones of *D. pulex* may develop morphological changes when predators are present but not when they are absent from the pond. Some of these changes are of such magnitude that they have been described as separate species. *D. minnehaha* is a morphological variation of *D. pulex* which develops spines in response to the stimuli of predators (Krueger and Dodson, 1981). Different genotypes of *D. pulex* react in different ways to the predator (*Chaoborus*) factor and to temperature (Havel, 1985).

3.2 FOOD AND FEEDING

3.2.1 Both *D. pulex* and *D. magna* are well adapted to live in algal blooms, which are high in proteins and carbohydrates, where they feed on algae and bacteria. *D. magna* prefers bacteria to algae as food (Ganf, 1983; Hadas et al., 1983) while *D. pulex* uses bacteria as food only when algal biomass declines (Borsheim and Olsen, 1984). Food type and abundance affect the sensitivity of *Daphnia* to pollutants and their reproduction rate. Keating and Dagbusan (1986) showed that both *D. pulex* and *D. magna* fed diatoms were more tolerant of pollutants than those fed only green algae. Lipid reserves are a good indication of the nutritional condition of the animals (Holm and Shapiro, 1984; Tessier and Goulden, 1982).

3.3 LIFE HISTORY

3.3.1 Four distinct periods may be recognized in the life history of *Daphnia*: (1) egg, (2) juvenile, (3) adolescence, and (4) adult (Pennak, 1989). The life span of *Daphnia*, from the release of the egg into the brood chamber until the death of the adult, is highly variable depending on the species and environmental conditions (Pennak, 1989). Generally the life span increases as temperature decreases, due to lowered metabolic activity. The average life span of *D. magna* is about 40 days at 25°C, and about 56 days at 20°C. The average life span of *D. pulex* at 20°C is approximately 50 days. Typically, a clutch of 6 to 10 eggs is released into the brood chamber, but as many as 57 have been reported. The eggs hatch in the brood chamber and the juveniles, which are already similar in form to the adults, are released in approximately two days when the female molts (casts off her exoskeleton or carapace). The

time required to reach maturity (produce their first offspring) in *D. pulex* varies from six to 10 days (mean = 7.78 days) and also appears to be dependent on body size. The growth rate of the organism is greatest during its juvenile stages (early instars), and the body size may double during each of these stages. *D. pulex* has three to four juvenile instars, whereas *D. magna* has three to five instars. Each instar stage is terminated by a molt. Growth occurs immediately after each molt while the new carapace is still elastic.

3.3.2 Following the juvenile stages, the adolescent period is very short, and consists of a single instar. It is during the adolescent instar that the first clutch of eggs reaches full development in the ovary. Generally, eggs are deposited in the brood chamber within minutes after molting, and the young which develop are released just before the next molt.

3.3.3 *D. magna* usually has 6-22 adult instars, and *D. pulex* has 18-25. In general, the duration of instars increases with age, but also depends on environmental conditions. A given instar generally lasts approximately two days under favorable conditions, but when conditions are unfavorable, may last as long as a week.

3.3.4 Four events take place in a matter of a few minutes at the end of each adult instar: (1) release of young from the brood chamber to the outside, (2) molting, (3) increase in size, and (4) release of a new clutch of eggs into the brood chamber. The number of young per brood is highly variable for *Daphnia*, depending primarily on food availability and environmental conditions. *D. magna* and *D. pulex* may both produce as many as 30 young during each adult instar, but more commonly the number is six to 10. The number of young released during the adult instars of *D. pulex* reaches a maximum at the tenth instar, after which there is a gradual decrease (Anderson and Zupancic, 1937). Scholtz et al. (1988) reported that nearly all of the eggs that are oviposited by *D. pulex* became neonates, indicating a highly successful hatching rate. The maximum number of young produced by *D. magna* occurs at the fifth adult instar, after which it decreases (Anderson and Jenkins, 1942).

4. CULTURING METHODS

4.1 SOURCES OF ORGANISMS

4.1.1 *Daphnia* are available from commercial biological supply houses. Only a small number of organisms (20-30) are needed to start a culture. *D. pulex* is preferred over *D. magna* by some biologists because it is more widely distributed, is tolerant of a wider range of environmental conditions, and is easier to culture. However, the neonates are smaller, swim faster and are more difficult to count, and produce more "floaters" than *D. magna* and, therefore, are somewhat more difficult to use in toxicity tests. Guidance on the source and species of *Daphnia* to be used by a permittee for effluent toxicity tests should be obtained from the permitting authority.

4.1.2 Cultures of test organisms should be started at least three weeks before the brood animals are needed, to ensure an adequate supply of neonates for the test.

4.1.3 Starter animals may be obtained from an outside source by shipping in polyethylene bottles. Approximately 20-30 animals and 3 mL of food (see below) are placed in a 1-L bottle filled full with culture water. Animals received from an outside source should be transferred to new culture media gradually over a period of 1-2 days to avoid mass mortality.

4.1.4 It is best to start the cultures with one animal, which is sacrificed after producing young, embedded, and retained as a permanent microscope slide mount to facilitate identification and permit future reference. The species identification of the stock culture should be verified by a taxonomic authority. The following procedure is recommended for making slide mounts of *Daphnia* (Beckett and Lewis, 1982):

1. Pipet the animal onto a watch glass.
2. Reduce the water volume by withdrawing excess water with the pipet.
3. Add a few drops of carbonated water (club soda or seltzer water) or 70% ethanol to relax the specimen so that the post-abdomen is extended. (Optional: with practice, extension of the postabdomen may be accomplished by putting pressure on the cover slip).
4. Place a small amount (one to three drops) of mounting medium on a glass microscope slide. The recommended mounting medium is CMCP-9/9AF Medium, prepared by mixing two parts of CMCP-9 with one part of CMCP-9AF. For more viscosity and faster drying, CMC-10 stained with acid fuchsin may be used. CMCP-9 and 9AF are available from Polysciences, Inc., Paul Valley Industrial Park, Warrington, PA 18976 (215-343-6484).
5. Using a forceps or a pipet, transfer the animal to the drop of mounting medium on the microscope slide.
6. Cover with a cover slip and exert minimum pressure to remove any air bubbles trapped under the cover slip. Slightly more pressure will extend the postabdomen.
7. Allow mounting medium to dry.
8. Make slide permanent by placing CMC-10 around the edges of the coverslip.
9. Identify to species (see Pennak, 1989).
10. Label with waterproof ink or diamond pencil.
11. Store for permanent record.

4.2 CULTURE MEDIA

4.2.1 Although *Daphnia* stock cultures can be successfully maintained in some tap waters, well waters, and surface waters, use of synthetic water as the culture medium is recommended because (1) it is easily prepared, (2) it is of known quality, (3) it yields reproducible results, and (4) allows adequate growth and reproduction. Reconstituted hard water (total hardness of 160 -180 mg/L as $CaCO_3$) is recommended for *D. magna* culturing, and reconstituted moderately hard water (total hardness of 80-90 mg/L $CaCO_3$) is recommended for *D. pulex* culturing. The quality of the dilution water is important in *Daphnia* culture. The use of MILLIPORE MILLI-Q® or SUPER-Q®, or equivalent, to prepare reconstituted water is highly recommended. The use of diluted mineral water (DMW) for culturing and testing is widespread due to the ease of preparation.

4.2.2 The chemicals used and instructions for preparation of reconstituted water are given in Section 7, Dilution Water. The compounds are dissolved in distilled or deionized water and the media are vigorously aerated for several hours before using. The initial pH of the media is between 7.0 and 8.0, but it will rise as much as 0.5 unit after the test is underway.

4.3 CULTURE CONDITIONS

4.3.1 *Daphnia* can be cultured successfully over a wide range of temperatures, but should be protected from sudden changes in temperature, which may cause death. The optimum temperature is approximately 20°C, and if ambient laboratory temperatures remain in the range of 18-26°C, normal growth and reproduction of *Daphnia* can be maintained without special temperature control equipment. *D. magna* can survive when the DO concentration is as low as 3 mg/L but *D. pulex* does best when the DO concentration is above 5 mg/L. Therefore it is recommended that the DO concentration in the culture be maintained at 5 mg/L or above. Unless the cultures are too crowded or overfed, aeration is usually not necessary.

4.3.2 Illumination

4.3.2.1 The variations in ambient light intensities (10-20 $\mu E/m^2/s$, or 50-100 ft-c) and prevailing day/night cycles in most laboratories do not seem to affect *Daphnia* growth and reproduction significantly. However, a minimum of 16 h of illumination should be provided each day.

4.3.3 Culture Vessels

4.3.3.1 Culture vessels of clear glass are recommended since they allow easy observation of the *Daphnia*. A practical culture vessel is an ordinary 4-L glass beaker, which can be filled with approximately 3 L medium (reconstituted water). Maintain several (at least five) culture vessels, rather than only one. This will ensure back-up cultures so that in the event of a population "crash" in one or several chambers, the entire *Daphnia* population will not be lost. If a vessel is stocked with 30 adult *Daphnia*, it will provide approximately 300 young each week.

4.3.3.2 Initially, all culture vessels should be washed well (see Section 5, Facilities and Equipment). After the culture is established, clean each chamber weekly with distilled or deionized water and wipe with a clean sponge to rid the vessel of accumulated food and dead *Daphnia* (see section on culture maintenance below). Once per month, wash each vessel with detergent during medium replacement. Rinse three times with tap water and then with culture medium to remove all traces of detergent.

4.3.4 Weekly Culture Media Replacement

4.3.4.1 Careful culture maintenance is essential. The medium in each stock culture vessel should be replaced three times each week with fresh medium.

This is best accomplished by changing solutions Monday, Wednesday, and Friday, as follows:

1. Place about 300 mL of the old media in a temporary holding vessel.
2. Transfer about 25 or 30 adults from the old culture vessel to the holding vessel using a wide bore pipette.
3. Discard the remaining *Daphnia* along with the media.
4. Clean the culture vessel as described above.
5. Fill the newly-cleaned vessel with fresh medium.
6. Gently transfer (by pouring) the contents of the temporary holding vessel (old medium with the *Daphnia*) into the vessel containing the new medium making sure that none of the animals stick to the sides of the vessel.
7. Feed the animals

4.3.4.2 If the medium is not replaced three times weekly, waste products will accumulate, which could cause a population crash or the production of males and/or sexual eggs.

4.3.4.3 *Daphnia* cultures should be thinned whenever the population exceeds 200 individuals per stock vessel to prevent over-crowding, which may cause a population crash, or the production of males and/or ephippia. A good time to thin the populations is on Monday, Wednesday, and Friday, before feeding. To transfer *Daphnia*, use a 15-cm disposable, jumbo bulb pipette, or 10-mL "serum" pipette which has had the delivery tip cut off and fire polished. The diameter of the opening should be approximately 5 mm. A serum pipette, a pipette bulb, such as a PROPIPETTE®, or (MOPET®) portable, motorized pipettor, will provide the controlled suction needed when selectively collecting *Daphnia*.

4.3.4.4 Liquid containing adult *D. pulex* and *D. magna* can be poured from one container to another without risk of air becoming trapped under their carapaces. However, the very young *Daphnia* are much more susceptible to air entrapment and for this reason should be transferred from one container to another using a pipette. The tip of the pipette should be kept under the surface of the liquid when the *Daphnia* are released.

4.3.4.5 Each culture vessel should be covered with a clear plastic sheet or glass plate to exclude dust and dirt, and minimize evaporation.

4.4 FOOD PREPARATION AND FEEDING

4.4.1 Feeding the proper amount of the right food is extremely important in *Daphnia* culturing. The key is to provide sufficient nutrition to support normal reproduction without adding excess food which may reduce the toxicity of the test solutions, clog the animal's filtering apparatus, or greatly decrease the DO concentration and increase mortality. YCT, a combination of Yeast, CEROPHYLL®, and Trout chow (or flake food), along with the unicellular green alga, *Selenastrum capricornutum*, will provide suitable nutrition if fed daily.

4.4.2 The YCT and algae are prepared as follows:

4.4.2.1 Digested trout chow (or flake food):

1. The preparation requires one week. Use starter or No. 1 pellets prepared according to current U.S. Fish and Wildlife Service specifications, or flake food. Suppliers of trout chow include Zeigler Bros., Inc., P.O. Box 95, Gardners, PA 17324 (717-780-9009); Glencoe Mills, 1011 Elliott, Glencoe, MN 55336 (612-864-3181); and Murray Elevators, 118 West 4800 South, Murray, UT 84107 (800-521-9092).
2. Add 5.0 g of trout chow pellets or flake food to 1 L of MILLI-Q® water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation is replaced during digestion. Because of the offensive odor usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.
3. At the end of digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (i.e., NITEX® 110 mesh). Combine with equal volumes of supernatant from CEROPHYLL® and yeast preparations (below). The supernatant can be used fresh, or frozen until use. Discard the sediment.

4.4.2.2 Yeast:

1. Add 5.0 g of dry yeast, such as FLEISCHMANN'S® to 1 L of MILLI-Q® water.
2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.
3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow (above) and CEROPHYLL® preparations (below). Discard excess material.

4.4.2.3 CEROPHYLL® (Dried, Powdered, Cereal Leaves):

1. Place 5.0 g of dried, powdered, cereal leaves in a blender. (Available as "CEREAL LEAVES," from Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 (800-325-3010); or as CEROPHYLL®, from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692-9012 (716-359-2502). Dried, powdered, alfalfa leaves obtained from health food stores have been found to be a satisfactory substitute for cereal leaves.
2. Add 1 L of MILLI-Q® water.
3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.
4. If a blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow to settle for 1 h. Decant the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard excess material.

4.4.2.4 Combined YCT Food:

1. Mix equal (approximately 300 mL) volumes of the three foods as described above.
2. Place aliquots of the mixture in small (50-mL to 100-mL) screw-cap plastic bottles and freeze until needed.
3. Freshly prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings, and is used for a maximum of one week.
4. It is advisable to measure the dry weight of solids in each batch of YCT before use. The food should contain 1.7- 1.9 g solids/L. Cultures or test solutions should contain 12-13 mg solids/L.

4.4.3 Algal (*Selenastrum*) Food

4.4.3.1 Algal Culture Medium

1. Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.
2. Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q® water. Mix well after the addition of each solution. Dilute to 1 L and mix well. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.
3. Immediately filter the medium through a 0.45 μm pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.
4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels.
5. Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

4.4.3.2 Algal Cultures

4.4.3.2.1 Two types of algal cultures are maintained: (1) stock cultures, and, (2) "food" cultures.

4.4.3.2.2 Establishing and Maintaining Stock Cultures of Algae

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring one milliliter to each of several 250-mL culture flasks containing 100 mL algal culture medium (prepared as described above). The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.
2. The stock cultures are used as a source of algae to initiate "food" cultures for *Daphnia* toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal food required for the *Daphnia* cultures and tests. Stock culture volume may

TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES.

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 ML MILLI-Q® WATER
1. MACRONUTRIENTS		
A.	$MgCl_2.6H_2O$	6.08 g
	$CaCl_2.2H_2O$	2.20 g
	$NaNO_3$	12.75 g
B.	$MgSO_4.7H_2O$	7.35 g
C.	K_2HPO_4	0.522 g
D.	$NaHCO_3$	7.50 g
2. MICRONUTRIENTS		
	H_3BO_3	92.8 mg
	$MnCl_2.4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg[a]
	$FeCl_3.6H_2O$	79.9 mg
	$CoCl_2.6H_2O$	0.714 mg[b]
	$Na_2MoO_4.2H_2O$	3.63 mg[c]
	$CuCl_2.2H_2O$	0.006 mg[d]
	$Na_2EDTA.2H_2O$	150.0 mg
	Na_2SeO_4	1.196 mg[e]

[a] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.

[b] $CoCl_2.6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to stock #1.

[c] $Na_2MoO_4.2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to stock #1.

[d] $CuCl_2.2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock #1.

[e] Na_2SeO_4 - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.

TABLE 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

MACRONUTRIENT	CONCENTRATION (MG/L)	ELEMENT	CONCENTRATION (MG/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2.6H_2O$	12.2	Mg	2.90
$CaCl_2.2H_2O$	4.41	Ca	1.20
$MgSO_4.7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

MICRONUTRIENT	CONCENTRATION (μG/L)	ELEMENT	CONCENTRATION (μG/L)
H_3BO_3	185	B	32.5
$MnCl_2.4H_2O$	416	Mn	115
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2.6H_2O$	1.43	Co	0.354
$CuCl_2.2H_2O$	0.012	Cu	0.004
$Na_2MoO_4.2H_2O$	7.26	Mo	2.88
$FeCl_3.6H_2O$	160	Fe	33.1
$Na_2EDTA.2H_2O$	300	--	----
Na_2SeO_4	2.39	Se	1.00

be rapidly "scaled up" to several liters, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.

3. Culture temperature is not critical. Stock cultures may be maintained in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).
4. Cultures are mixed twice daily by hand or stirred continuously.
5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three milliliters of 7-day old algal stock culture, containing approximately 1.5 X 10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 1,000-30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.
6. Stock cultures should be examined microscopically weekly, at transfer, for microbial contamination. Reserve quantities of culture organisms can be maintained for 6-12 months if stored in the dark at 4°C. It is advisable to prepare new stock cultures from "starter" cultures obtained from established outside sources of organisms every four to six months.

4.4.3.2.3 Establishing and Maintaining "Food" Cultures of Algae

1. "Food" cultures are started seven days prior to use for *Daphnia* cultures and tests. Approximately 20 mL of 7-day-old algal stock culture (described in the previous paragraph), containing 1.5 X 10^6 cells/mL, are added to each liter of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of approximately 30,000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are terminated in 7-10 days. A one-month supply of algal food can be grown at one time, and the excess stored in the refrigerator.
2. Food cultures may be maintained at 25°C in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).
3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) or in a moderately aerated separatory funnel, or are mixed twice daily by hand. If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees. Caution should be exercised to prevent the culture temperature from rising more than 2-3°C.

4.4.3.3 Preparing Algal Concentrate for Use as *Daphnia* Food

1. An algal concentrate containing 3.0 to 3.5 X 10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a

refrigerator for approximately two-to-three weeks and siphoning off the supernatant.

2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer, and used to determine the concentration required to achieve a final cell count of 3.0 to 3.5 X 10^7/mL.
3. Assuming a cell density of approximately 1.5 X 10^6 cells/mL in the algal food cultures at 7 days, and 100% recovery in the concentration process, a 3-L, 7-10 day culture will provide 4.5 X 10^9 algal cells. This number of cells would provide approximately 150 mL of algal cell concentrate.
4. Algal concentrate may be stored in the refrigerator for one month.

4.5 FEEDING

4.5.1 Feeding rate and frequency are important in maintaining the organisms in optimal condition so that they achieve maximum reproduction. Stock cultures which are stressed because they are not adequately fed may produce low numbers of young, large numbers of males, and ephippial females. When the young taken from these inadequately fed *Daphnia* cultures are used in toxicity tests, they may show higher than acceptable mortality in controls and greater than normal sensitivity to toxicants. Steps to follow when feeding the YCT and algal diet are as follows:

1. If YCT is frozen, remove a bottle of the food from the freezer at least 1 h before feeding time, and allow to thaw.
2. Mass cultures are fed Monday, Wednesday, and Friday at the rate of 4.5 mL YCT and 2 mL of algae concentrate per 3-L culture.
3. On Tuesday and Thursday the culture water is stirred to re-suspend the settled algae and another 2 mL of algal concentrate is added.
4. The YCT and algal concentrate is thoroughly mixed by shaking before dispensing.
5. Return unused YCT food mixture and algal concentrate to the refrigerator. Do not re-freeze the YCT. Discard unused portion of YCT after one week.

4.5.2 The quality of food prepared with newly acquired supplies of yeast, trout chow, and dried cereal leaves, or algae, should be determined in side-by-side comparisons of *Daphnia* survival and reproduction tests, using the new food and food of known, acceptable quality, over a seven-day period in control medium.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.3. MYSIDS (*MYSIDOPSIS BAHIA*)

1. DISTRIBUTION

1.1 Mysids (Figure 1) are small shrimp-like crustaceans found in both the marine and freshwater environments. The mysid that currently is of primary interest in the NPDES program is the estuarine species, *Mysidopsis bahia*. It occurs primarily at salinities above 15‰. Stuck et al. (1979a) and Price (1982) found it in greatest abundance at salinities near 30‰. Three sympatric species of *Mysidopsis*, *M. almyra*, *M. bahia*, and *M. bigelowi*, have been cultured and used in toxicity testing. The distribution of *Mysidopsis* species has been reported by Stuck et al. (1979b), Price (1982), and Heard et al. (1987).

1.2 Other marine mysids that have been used in toxicity testing and held or cultured in the lab include *Metamysidopsis elongata*, *Neomysis americana*, *Neomysis awatschensis*, *Neomysis intermedia*, and recently for the Pacific coast, *Holmesimysis sculpta* and *Neomysis mercidis*. A freshwater species, *Mysis relicta*, presently not used in toxicity testing, but found in the same habitat as *Daphnia pulex*, might be considered in the future for toxicity testing.

2. LIFE CYCLE

2.1 In laboratory culture, *Mysidopsis bahia* reach sexual maturity in 12 to 20 days, depending on water temperature and diet (Nimmo et al., 1977). Normally, the female will have eggs in the ovary at approximately 12 days of age. The lamellae of the marsupium pouch have formed or are in the process of forming when the female is approximately 4 mm in length (Ward, 1993). Unlike *Daphnia*, the eggs will not develop unless fertilized. Mating takes place at night and lasts only a few minutes (Mauchline, 1980).

2.2 Brood pouches are normally fully formed at approximately 15 days (approximately 5 mm in body length), and young are released in 17 to 20 days (Ward, 1993). The number of eggs deposited in the brood and the number of young produced per brood are a direct function of body length as well as environmental conditions. Mature females have produced as many as 25 Stage I larvae (egg-shaped embryo) per brood (8-9 mm in body length) in natural and artificial seawater (FORTY FATHOMS®) but average 11 ± 6 Stage III larvae (final stage before larvae are released), with increasing numbers correlated with increasing body length (Ward, 1993). A new brood is produced every 4 to 7 days.

2.3 At time of emergence, juveniles are immobile, making them susceptible to predation by adult mysids. The juveniles are planktonic for the first 24-48 hours and then settle to the bottom, orient to the current, and actively pursue food organisms such as *Artemia*. Carr et al. (1980) reported that the

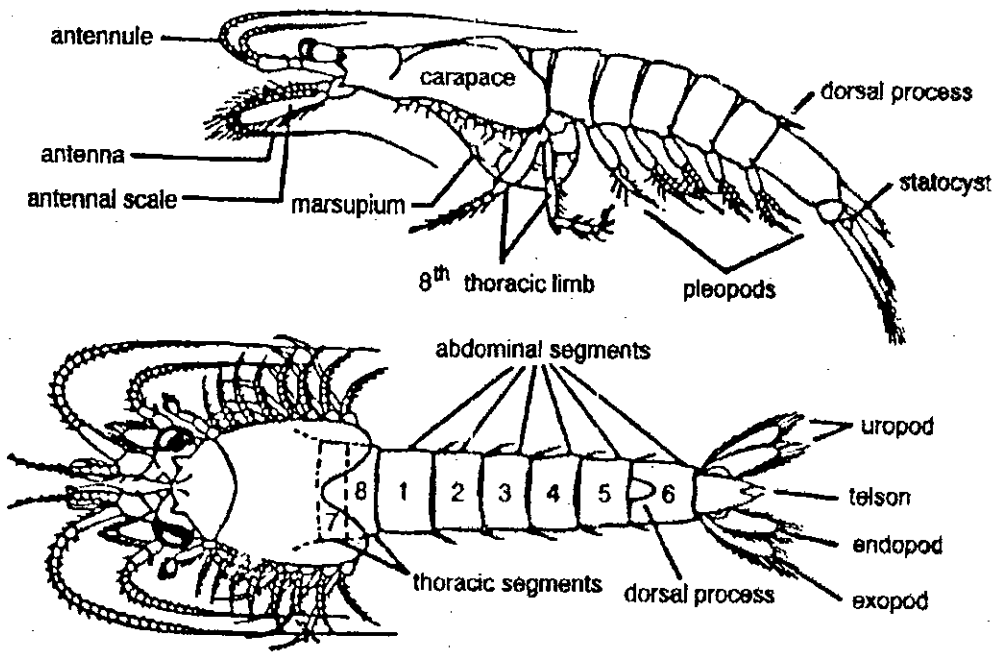

Figure 1. Lateral and dorsal view of a mysid with morphological features identified (From Stuck et al., 1979a).

stage in the life cycle of *M. almyra* most sensitive to drilling mud was the juvenile molt, which occurs between 24 and 48 hours after release from the brood pouch. Ward (1989) found a relationship between $CaCO_3$ level and growth and reproduction and that *M. bahia* were more sensitive to cadmium during molting (24-72 h post release) in high or low levels of $CaCO_3$. Work done by Lee and Buikema (1979) for *Daphnia pulex* also showed increased sensitivity during molting.

3. MORPHOLOGY AND TAXONOMY

3.1 Since *Mysidopsis bahia* occur with two other species of *Mysidopsis*, an understanding of the taxonomy of *M. almyra*, *M. bahia*, and *M. bigelowi* is

important for culturing and testing practices. The taxonomic key of Heard et al. (1987) is suggested (see Table 1 for morphological guide to *Mysidopsis*).

3.2 Adults of *M. bahia* range in length from 4.4 mm to 9.4 mm (Molenock, 1969), measured from the anterior margin of the carapace to the end of uropods. The mature females are normally larger than the males and the pleopods of the female are smaller than those of the male (Ward, 1993) (Figure 2). *Mysidopsis bahia* can be positively identified as male or female when they are 4 mm in body length (Ward, 1993). Living organisms are usually transparent, but may be tinted yellow, brown or black. *Mysidopsis bigelowi* can be readily distinguished from *M. almyra* and *M. bahia* by the morphology of the second thoracic leg. *Mysidopsis bigelowi* has a greatly enlarged endopod of the thoracic limb 2 ("first leg") and the limb has a distinctive row of 6 to 12 spiniform setae on the inner margin of the sixth segment (Heard et al., 1987). *Mysidopsis bahia* can also be distinguished from other species of *Mysidopsis* by the number of apical spines on the telson (4-5 pairs) and the number of spines on the inner uropods distal to the statocyst (normally 2-3) (Figure 2).

3.3 Heard et al. (1987) state that the most reliable character for separating adult *M. almyra* and *M. bahia* is the number of spines on the inner uropods (*M. almyra* will always have a single spine). Further, Price (1982) found that for all stages of development for both species, the shape of the anterior margin of the carapace (rostral plate) could be used to distinguish *M. almyra* (broadly rounded) from *M. bahia* (more produced). Figure 2 illustrates the morphological features most useful in identifying *M. bahia* (redrawn from Molenock, 1969; Heard et al., 1987).

4. CULTURE METHODS

4.1 SOURCE OF ORGANISMS

4.1.1 Starter cultures of mysids can be obtained from commercial sources, particularly in the Gulf of Mexico region for *M. almyra* and *M. bahia*.

4.1.2 Mysids of different species can also be collected by plankton tows or dip nets (approximately 1.0 mm mesh size) in estuarine systems. Heard et al. (1987) have identified specimens of *M. bahia* along the eastern coast, however, it has been principally identified as a subtropical species found in the Gulf of Mexico and along the east coast of Florida. Since many species of mysids may be present at a given collection site, the identification of the organisms selected for culture should be verified by an experienced taxonomist. The permittee should consult the permitting authority for guidance on the source of test organisms (indigenous or laboratory reared) before use.

4.2 CULTURING SYSTEM

4.2.1 Stock cultures can be maintained in continuous-flow or closed recirculating systems. In laboratory culture of *M. bahia*, recirculating systems are probably the most common practice. During the past ten years, a number of closed recirculating systems have been described. Since no single recirculating technique is the best in all respects, the system adopted will

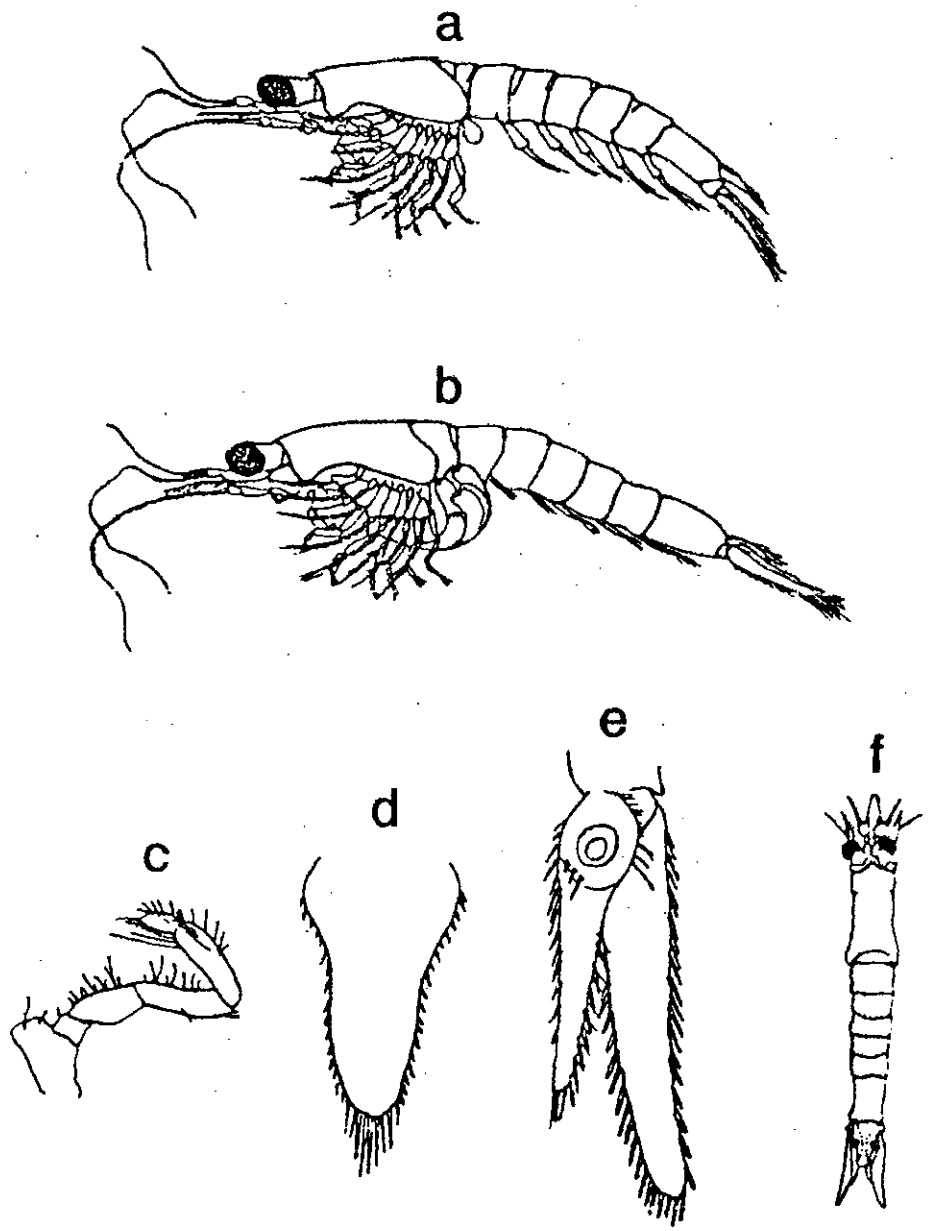

Figure 2. Morphological features most useful in identifying *Mysidopsis bahia*. a. male; b. female; c. thoracic leg 2; d. telson; e. right uropod, dorsal; f. male, dorsal (redrawn from Molenock, 1969; Heard et al., 1987). Note gonad in area where marsupium is located on female and length of male pleopods as compared to female. Also note the 3 spines on the endopod of the uropod (e).

TABLE 1. GENUS *MYSIDOPSIS*: COMMON AND SPECIFIC CHARACTERS OF SPECIES KNOWN FROM U.S. ATLANTIC AND GULF OF MEXICO WATERS (HEARD, ET AL., 1987 AND MODIFIED IN PART FROM BRATTEGARD, 1969)

	M. eclipes	M. brattstroemi	M. mortenseni	M. furca	M. almyra	M. bahia	M. bigelowi	M. sp. (Inshore)	M. sp. (Offshore)
Anterior dorsal margin of carapace	Triangular	Triangular	Triangular	Rounded	Rounded	Triangular	Triangular	Triangular	Triangular
Presence of distal segment on antennal	yes	yes	yes	yes	no	no	no	no	no
Length/breadth ratio of antennal scale	2-2.5	3-3.5	3.5-4	4	6	6	5.4	5.4	5.2
# segments in carpopropodus of thoracic endopods 3-8	3	3	3 (2 in juv.)	3	2	2	2	2	2
# segments in exopod of male pleopod 4	?	6	6-7	7	7	7	7-8	7	7
# spines on uropodal endopod	8-9	10-20	18-31	20-40	1	1-4	5 (occ. 3-4)	3-4 (occ. 2 or 5)	5 (occ. 4)
Length of terminal pair(s) of telson spines relative to lateral margin spines	Almost twice as long	Slightly longer	Slightly longer	> twice as long	Gradually increasing	Gradually increasing	Abruptly increasing	Abruptly increasing	Abruptly increasing
# of pairs of apical telson spines	1	1	1	2	4-8	3-6	3	3-4	3
# setae on inner margin of segment 6 of second thoracic endopod	ca 3	ca 4	ca 3	ca 3	2-3	2-3	6-12	6-12	6-12
# setae on inner margin of segment 5 of second thoracic endopod	ca 5	ca 4	ca 2	ca 3	7-18	7-18	2	2	5-7

depend on the facilities and equipment available and the objectives of the culturing activities. Two other species of mysid, *M. almyra* and *M. bigelowi*, have also been successfully reared in the system described in this section (Ward, 1991). Further, there now exist a number of review papers (Venables, 1987; and Lussier et al., 1988) that describe in detail techniques developed by others that will be very helpful in culturing *Mysidopsis*.

4.2.2 Closed recirculating systems are unique because the re-used seawater they contain develops an unusual set of characteristics caused primarily by metabolic waste produced by the mysids. The accumulation of waste products and suspended particles in the water column is prevented by passing the seawater through a biological filtration system, in which ammonia and nitrite are oxidized by nitrifying bacteria.

4.3 CULTURE TANKS

4.3.1 Stock cultures of mysids are maintained in a closed recirculating system. The system should consist of four 200-L glass aquaria. However, smaller tanks, such as 80-L glass aquaria, can be used. When setting up a system, it is important to consider surface to volume ratio since this will determine how many mysids can be held in each aquarium. If smaller tanks must be used, the 20-gallon "high" form is recommended. Figure 3 (Ward, 1984; 1991) illustrates the main components of the biological filtration system. The flow rate through the filter is controlled by the water valve and is maintained between 4-5 L/min. This flow will be sufficient to establish a moderate current (from the filter return line) in the aquarium to allow the mysids (which are positively rheotactic) to align themselves with the current formed.

4.3.2 The filtration system consists of commercially-available under-gravel filter plates and external power filter. Each aquarium has two filter plates, forming a false bottom on each side of the tank, on which 2 cm of crushed coral are placed. The external power filter (Eheim, model 2017) canister is layered as shown in Figure 3 with a thin layer of filter fiber between each layer of carbon and crushed oyster shells. There has been some modification of the original filtration system (Ward, 1984), with crushed coral instead of oyster shells used on the filter bed, because crushed coral does not dissolve in seawater as readily as crushed oyster shells. If the system described above cannot be used, an acceptable alternative is an airlift pumping arrangement (Spotte, 1979). Crushed coral and oyster shells are commercially available and should be washed with deionized water and autoclaved before use.

4.4 CULTURE MEDIA

4.4.1 A clean source of filtered natural seawater (0.45 μm pore diameter) should be used to culture *Mysidopsis bahia*, however, artificial seasalts (FORTY FATHOMS®) have also been successfully used (Ward, 1993). A salinity range between 20 and 30‰ can be used (25‰ is suggested) to culture *M. bahia*. Leger and Sorgeloos (1982) reported success in culturing *M. bahia* in a formula following Dietrich and Kalle (Kalle, 1971), and still report continued use of this formula (Leger et al., 1987b). Other commercial brands have also been used (Reitsema and Neff, 1980; Nimmo and Iley, 1982; Nimmo et al., 1988) with

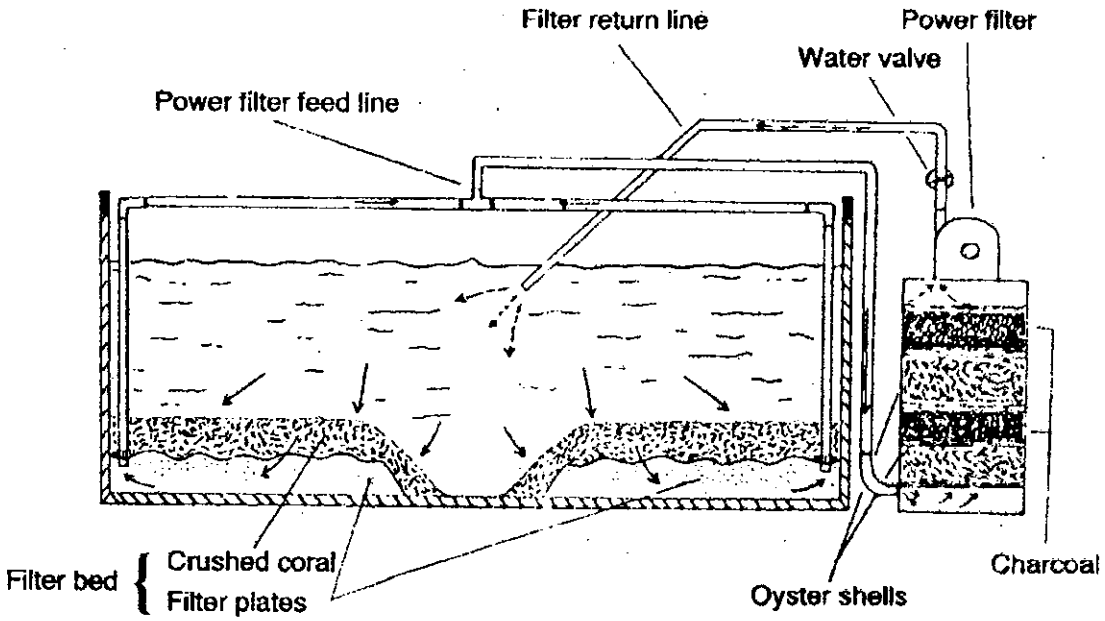

Figure 3. Closed recirculating system showing the two phases of the biological filtration system which consists of the filter bed and external power filter (from Ward, 1984; Ward, 1991).

varying degrees of success. The culture methods presented in Ward (1984; 1991) have been tried with a number of commercial brands of artificial seawater listed in Bidwell and Spotte (1985). Commercial brands of seasalts can be extremely variable in the amount of $NaHCO_3$ they provide, which, if not controlled, can affect growth and reproduction (Ward; 1989, 1991). In a comparative study, Ward (1993) found normal larval development within the marsupium using both natural seawater and FORTY FATHOMS® (i.e., Stage I - embryo; Stage II - eyeless larva; Stage III - eyed larva which is the final stage before release) and stressed the importance of proper preparation of the seasalts and monitoring of conditions in the tank.

4.4.2 The culture media should be aged to allow the build-up of nitrifying bacteria in the filter substrate. To expedite the aging process, 15 mL of a concentrated suspension of *Artemia* should be added daily. If using natural or artificial seawater, the carbonate alkalinity level should be maintained between 90 and 120 mg/L. It is also important to establish an algal community, *Spirulina subsalsa*, in the filter bed (Ward, 1984) and a healthy surface dwelling diatom community, *Nitzchia* sp., on the walls (Ward, 1991) in conjunction with the transfer of part of the biological filter from a healthy tank, when possible. After seven days, the suitability of the medium is checked by adding 20 adult mysids. If the organisms survive for 96 hours, the culture should be suitable for stocking.

4.4.3 If brine solutions are used, 100‰ salinity must not be exceeded. This corresponds to a carbonate alkalinity value of approximately 50 mg/L, which will allow relatively normal physiological mechanisms associated with $CaCO_3$ to occur during certain phases of the life cycle for *M. bahia* (Ward, 1989).

4.5 ENVIRONMENTAL FACTORS

4.5.1 Temperature must be maintained within a range of 24°C to 26°C. Twelve to sixteen hours illumination should be provided daily at 50 to 100 ft-c. The daily light cycle can be provided by combining overhead room lights, cool-white fluorescent bulbs (approx. 50 ft-c, 12L:12D), with individual Grow-lux fluorescent bulbs placed horizontally over each tank (approx. 65 ft-c, 10L:14D). This procedure will avoid acute illumination changes by allowing the room lights to turn on one hour before and one hour after the aquaria lights. A timing device, such as an electronic microprocessor-based timer (CHRONTROL®, model CD, or equivalent) can be used to control the light cycle. These procedures are fully outlined in Ward (1984; 1991).

4.5.2 Good aeration (≥60% saturation by vigorous aeration with an air stone), a 10-20 percent exchange of seawater per week, and carbonate in the filtration system are essential in helping to control pH drops caused by oxidation of NH_4-N and NO_2-N by bacteria.

4.5.3 The single most important environmental factor when culturing *Mysidopsis bahia* or other organisms in recirculators is the conversion of ammonia to nitrite, and nitrite to nitrate by nitrifying bacteria. Spotte (1979) has suggested upper limits of 0.1 mg total NH_4-N/L, 0.1 mg NO_2-N/L and 20 mg NO_3-N/L for good laboratory operation of recirculating systems. For the recirculating system and techniques described here for mysids, the levels of