

Method 1003.0: Green Alga, *Selenastrum capricornutum*, Growth Test; Chronic Toxicity

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**Short-term Methods for Estimating the Chronic Toxicity of
Effluents and Receiving Waters to Freshwater Organisms**

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SECTION 14

TEST METHOD

GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, GROWTH TEST METHOD 1003.0

14.1 SCOPE AND APPLICATION

14.1.1 This method measures the chronic toxicity of effluents and receiving water to the freshwater green alga, *Selenastrum capricornutum*, in a four-day static test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

14.1.2 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

14.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly degradable or highly volatile toxicants present in the source may not be detected in the test.

14.1.4 This test method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

14.1.5 This test is very versatile because it can also be used to identify wastewaters which are biostimulatory and may cause nuisance growths of algae, aquatic weeds, and other organisms at higher trophic levels.

14.2 SUMMARY OF METHOD

14.2.1 A green alga, *Selenastrum capricornutum*, population is exposed in a static system to a series of concentrations of effluent, or to receiving water, for 96 h. The response of the population is measured in terms of changes in cell density (cell counts per mL), biomass, chlorophyll content, or absorbance.

14.3 INTERFERENCES

14.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

14.3.2 Adverse effects of high concentrations of suspended and/or dissolved solids, color, and extremes of pH may mask the presence of toxic substances.

14.3.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.3.4 Pathogenic organisms and/or planktivores in the dilution water and effluent may affect test organism survival and growth, and confound test results.

14.3.5 Nutrients in the effluent or dilution water may confound test results.

14.4 SAFETY

14.4.1 See Section 3, Safety and Health.

14.5 APPARATUS AND EQUIPMENT

14.5.1 Laboratory *Selenastrum capricornutum* culture unit -- see culturing methods below and USEPA, 2002a. To test effluent toxicity, sufficient numbers of log-phase-growth organisms must be available.

14.5.2 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L or more.

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

14.5.4 Environmental chamber, incubator, or equivalent facility -- with "cool-white" fluorescent illumination ($86 \pm 8.6 \mu\text{E}/\text{m}^2/\text{s}$, $400 \pm 40 \text{ ft-c}$, or 4306 lux) and temperature control ($25 \pm 1^\circ\text{C}$).

14.5.5 Mechanical shaker -- capable of providing orbital motion at the rate of 100 cycles per minute (cpm).

14.5.6 Light meter -- with a range of $0\text{-}200 \mu\text{E}/\text{m}^2/\text{s}$ ($0\text{-}1000 \text{ ft-c}$).

14.5.7 Water purification system -- MILLIPORE MILLI-Q[®], deionized water or equivalent (see Section 5, Facilities, Equipment, and Supplies).

14.5.8 Balance -- analytical, capable of accurately weighing 0.00001 g.

14.5.9 Reference weights, class S -- for checking performance of balance.

14.5.10 Volumetric flasks and graduated cylinders -- class A, 10-1000 mL, borosilicate glass, for culture work and preparation of test solutions.

14.5.11 Volumetric pipets -- class A, 1-100 mL.

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

14.5.13 Pipet bulbs and fillers -- PROPIPET[®], or equivalent.

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

14.5.15 Test chambers -- four 125 or 250 mL borosilicate, Erlenmeyer flasks, with foam plugs or stainless steel or Shumadzu closures. For special glassware cleaning requirements (see Section 5, Facilities, Equipment, and Supplies).

14.5.16 Culture chambers -- 1-4 L borosilicate, Erlenmeyer flasks.

14.5.17 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

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

14.5.19 Thermometer, National Bureau of Standards Certified, (see USEPA Method 170.1, USEPA, 1979b) -- to calibrate laboratory thermometers.

14.5.20 Meters, pH and specific conductivity -- for routine physical and chemical measurements.

14.5.21 Tissue grinder -- for chlorophyll extraction.

14.5.22 Fluorometer (Optional) -- equipped with chlorophyll detection light source, filters, and photomultiplier tube (Turner Model 110 or equivalent).

14.5.23 UV-VIS spectrophotometer -- capable of accommodating 1-5 cm cuvettes.

14.5.24 Cuvettes for spectrophotometer -- 1-5 cm light path.

14.5.25 Electronic particle counter (Optional) -- Coulter Counter, Model ZBI, or equivalent, with mean cell (particle) volume determination.

14.5.26 Microscope -- with 10X, 45X, and 100X objective lenses, 10X ocular lenses, mechanical stage, substage condenser, and light source (inverted or conventional microscope).

14.5.27 Counting chamber -- Sedgwick-Rafter, Palmer-Maloney, or hemocytometer.

14.5.28 Centrifuge -- with swing-out buckets having a capacity of 15-100 mL.

14.5.29 Centrifuge tubes -- 15-100 mL, screw-cap.

14.5.30 Filtering apparatus -- for membrane and/or glass fiber filters.

14.6 REAGENTS AND CONSUMABLE MATERIALS

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

14.6.2 Data sheets (one set per test) -- for recording data.

14.6.3 Tape, colored -- for labeling test chambers.

14.6.4 Markers, waterproof -- for marking containers, etc.

14.6.5 Reagents for hardness and alkalinity tests -- see USEPA Methods 130.2 and 310.1, USEPA, 1979b.

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

14.6.7 Specific conductivity standards (see USEPA Method 120.1, USEPA, 1979b).

14.6.8 Standard particles -- such as chicken or turkey fibroblasts or polymer microspheres, $5.0 \pm 0.03 \mu\text{m}$ diameter, $65.4 \mu\text{m}^3$ volume, for calibration of electronic particle counters.

14.6.9 Membranes and filling solutions for DO probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

14.6.10 Laboratory quality control samples and standards -- for calibration of the above methods.

14.6.11 Reference toxicant solutions -- see Section 4, Quality Assurance.

14.6.12 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

14.6.13 Effluent or receiving water and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Testing.

14.6.14 Acetone -- pesticide-grade or equivalent.

14.6.15 Dilute (10%) hydrochloric acid -- carefully add 10 mL of concentrated HCl to 90 mL of MILLI-Q® water.

14.6.16 TEST ORGANISMS, GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*

14.6.16.1 *Selenastrum capricornutum*, a unicellular coccoid green alga, is the test organism. The genus and species name of this organism was formally changed to *Pseudokirchneriella subcapitata* (Hindak, 1990), however, the method manual will continue to refer to *Selenastrum capricornutum* to maintain consistency with previous versions of the method.

14.6.16.2 Algal Culture Medium is prepared as follows:

14.6.16.2.1 Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.

14.6.16.2.2 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, mix well, and adjust the pH to 7.5 ± 0.1 , using 0.1N NaOH or HCl, as appropriate. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.

14.6.16.2.3 Immediately filter the pH-adjusted 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.

14.6.16.2.4 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. If a 0.22 µg filter is used no sterilization is needed.

14.6.16.2.5 Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

14.6.16.2.6 When prepared according to Table 1, the micronutrient stock solution contains ethylenediaminetetraacetic acid (EDTA). EPA requires the addition of EDTA to nutrient stock solutions when conducting the *Selenastrum capricornutum* Growth Test and submitting data under NPDES permits. The use of EDTA improves test method performance by reducing the incidence of false positives and increasing test method precision. In interlaboratory testing of split samples analyzed with and without the addition of EDTA, false positive rates were 0.00% with EDTA and 33.3% without EDTA (USEPA, 2001a). Interlaboratory variability, expressed as the CV for IC25 values, was 34.3% with EDTA and 58.5% without EDTA (USEPA, 2001a). While the addition of EDTA improves test performance, EPA also cautions that the addition of EDTA may cause the *Selenastrum capricornutum* Growth Test to underestimate the toxicity of metals. Regulatory authorities should consider this possibility when selecting test methods for monitoring effluents that are suspected to contain metals. As recommended in EPA's Technical Support Document for Water Quality-Based Toxics Control (USEPA, 1991a), the most sensitive of at least three test species from different phyla should be used for monitoring the toxicity of effluents.

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 ₂ O | 6.08 | g |
| | CaCl ₂ ·2H ₂ O | 2.20 | g |
| | NaNO ₃ | 12.75 | g |
| B. | MgSO ₄ ·7H ₂ O | 7.35 | g |
| C. | K ₂ HPO ₄ | 0.522 | g |
| D. | NaHCO ₃ | 7.50 | g |
| 2. MICRONUTRIENTS | | | |
| | H ₃ BO ₃ | 92.8 | mg |
| | MnCl ₂ ·4H ₂ O | 208.0 | mg |
| | ZnCl ₂ | 1.64 | mg ¹ |
| | FeCl ₃ ·6H ₂ O | 79.9 | mg |
| | CoCl ₂ ·6H ₂ O | 0.714 | mg ² |
| | Na ₂ MoO ₄ ·2H ₂ O | 3.63 | mg ³ |
| | CuCl ₂ ·2H ₂ O | 0.006 | mg ⁴ |
| | Na ₂ EDTA·2H ₂ O | 150.0 | mg |
| | Na ₂ SeO ₄ | 1.196 | mg ⁵ |

¹ ZnCl₂ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

² CoCl₂·6H₂O - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

³ Na₂MoO₄·2H₂O - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock 2, micronutrients.

⁴ CuCl₂·2H₂O - 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 2, micronutrients.

⁵ Na₂SeO₄ - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

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 ₂ O | 12.2 | Mg | 2.90 |
| CaCl ₂ ·2H ₂ O | 4.41 | Ca | 1.20 |
| MgSO ₄ ·7H ₂ O | 14.7 | S | 1.91 |
| K ₂ HPO 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 ₃ BO ₃ | 185.0 | B | 32.5 |
| MnCl ₂ ·4H ₂ O | 416.0 | Mn | 115.0 |
| ZnCl ₂ | 3.27 | Zn | 1.57 |
| CoCl ₂ ·6H ₂ O | 1.43 | Co | 0.354 |
| CuCl ₂ ·2H ₂ O | 0.012 | Cu | 0.004 |
| Na ₂ MoO ₄ ·2H ₂ O | 7.26 | Mo | 2.88 |
| FeCl ₃ ·6H ₂ O | 160.0 | Fe | 33.1 |
| Na ₂ EDTA·2H ₂ O | 300.0 | -- | ---- |
| Na ₂ SeO ₄ | 2.39 | Se | 0.91 |

14.6.16.3 Stock Algal Cultures

14.6.16.3.1 See Section 6, Test Organisms, for information on sources of "starter" cultures of the green alga, *Selenastrum capricornutum*.

14.6.16.3.2 Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring 1 mL to a culture flask containing control algal culture medium (prepared as described above). The volume of stock culture medium initially prepared will depend upon the number of test flasks to be inoculated later from the stock, or other planned uses, and may range from 25 mL in a 125 mL flask to 2 L in a 4-L flask. 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.

14.6.16.3.3 Maintain the stock cultures at 25 ± 1 °C, under continuous "Cool-White" fluorescent lighting of 86 ± 8.6 $\mu\text{E}/\text{m}^2/\text{s}$ (400 ± 40 ft-c). Shake continuously at 100 cpm or twice daily by hand.

14.6.16.3.4 Transfer 1 to 2 mL of stock culture weekly to 50 - 100 mL of new culture medium to maintain a continuous supply of "healthy" cells for tests. Aseptic techniques should be used in maintaining the algal cultures, and extreme care should be exercised to avoid contamination. Examine the stock cultures with a microscope for contaminating microorganisms at each transfer.

14.6.16.3.5 Viable unialgal culture material may be maintained for long periods of time if placed in a refrigerator at 4°C.

14.6.16.4 It is recommended that chronic toxicity tests be performed monthly with a reference toxicant. Algal cells four to seven days old are used to monitor the chronic toxicity (growth) of the reference toxicant to the algal stock produced by the culture unit (see Section 4, Quality Assurance, Subsection 4.17).

14.6.16.5 Record Keeping

14.6.16.5.1 Records, kept in a bound notebook, include (1) dates culture media was prepared, (2) source of "starter" cultures, (3) date stock cultures were started, (4) cell density in stock cultures, and (5) dates and results of reference toxicant tests performed (see Section 4, Quality Assurance).

14.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

14.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

14.8 CALIBRATION AND STANDARDIZATION

14.8.1 See Section 4, Quality Assurance.

14.9 QUALITY CONTROL

14.9.1 See Section 4, Quality Assurance.

14.10 TEST PROCEDURES

14.10.1 TEST SOLUTIONS

14.10.1.1 Receiving Waters

14.10.1.1.1 The sampling point is determined by the objectives of the test. Receiving water toxicity is determined with samples used directly as collected or after samples are passed through a 60 μm NITEX® filter and compared

without dilution against a control. Using four replicate chambers per test, each containing 100 mL and 400 mL for chemical analyses, would require approximately 1 L or more of sample for the test.

14.10.1.2 Effluents

14.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of $\pm 100\%$, and testing of concentrations between 6.25% and 100% effluent using five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Improvements in precision decline rapidly if the dilution factor is increased beyond 0.5 and precision declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends using a ≥ 0.5 dilution factor.**

14.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions should be added at the lower range of the effluent concentrations.

14.10.1.2.3 The volume of effluent required for the test is 1 to 2 L. Sufficient test solution (approximately 900 or 1500 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses at the high, medium, and low test concentrations. There is no daily renewal of test solution.

14.10.1.2.4 Tests should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.10.1.2.5 Just prior to test initiation (approximately 1 h) the temperature of sufficient quantity of the sample to make test solutions should be adjusted to the test temperature and maintained at that temperature during the addition of dilution water.

14.10.1.2.6 The DO of the test solutions should be checked prior to test initiation. If any of the solutions are supersaturated with oxygen, all of the solutions and the control should be gently aerated. If or any solution has a DO concentration below 4.0 mg/L, all of the solutions and the control must be gently aerated.

14.10.1.2.7 Effluents may be toxic and/or nutrient poor. "Poor" growth in an algal toxicity test, therefore, may be due to toxicity or nutrient limitation, or both. To eliminate false negative results due to low nutrient concentrations, 1 mL of each stock nutrient solution is added per liter of effluent prior to use in preparing the test dilutions. Thus, all test treatments and controls will contain at a minimum the concentration of nutrients in the stock culture medium.

14.10.1.2.8 If samples contain volatile substances, the test sample should be added below the surface of the dilution water towards the bottom of the test container through an appropriate delivery tube.

14.10.1.3 Dilution Water

14.10.1.3.1 Dilution water may be stock culture medium, any uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other natural water (see Section 7, Dilution Water). However, if water other than the stock culture medium is used for dilution water, 1 mL of each stock nutrient solution should be added per liter of dilution water. Natural waters used as dilution water must be filtered through a prewashed filter, such as a GF/A, GF/C, or equivalent filter, that provides 0.45 μm particle size retention.

14.10.1.3.2 If the growth of the algae in the test solutions is to be measured with an electronic particle counter, the effluent and dilution water must be filtered through a GF/A or GF/C filter, or other filter providing 0.45 μm particle

size retention, and checked for "background" particle count before it is used in the test. Glass-fiber filters generally provide more rapid filtering rates and greater filtrate volume before plugging.

14.10.1.4 Preparation of Inoculum

14.10.1.4.1 The inoculum is prepared no more than 2 to 3 h prior to the beginning of the test, using *Selenastrum capricornutum* harvested from a four- to-seven-day stock culture. Each milliliter of inoculum must contain enough cells to provide an initial cell density of approximately 10,000 cells/mL ($\pm 10\%$) in the test flasks. Assuming the use of 250 mL flasks, each containing 100 mL of test solution, the inoculum must contain 1,000,000 cells/mL.

14.10.1.4.2 Estimate the volume of stock culture required to prepare the inoculum. As an example, if the four-to-seven-day-old stock culture used as the source of the inoculum has a cell density of 2,000,000 cells/mL, a test employing 24 flasks, each containing 100 mL of test medium and inoculated with a total of 1,000,000 cells, would require 24,000,000 cells or 15 mL of stock solution (24,000,000/2,000,000) to provide sufficient inoculum. It is advisable to prepare a volume 20% to 50% in excess of the minimum volume required, to cover accidental loss in transfer and handling.

14.10.1.4.3 Prepare the inoculum as follows:

1. Centrifuge 15 mL of stock culture at 1000 x g for 5 min. This volume will provide a 50% excess in the number of cells.
2. Decant the supernatant and resuspend the cells in 10 mL of control medium.
3. Repeat the centrifugation and decantation step, and resuspend the cells in 10 mL control medium.
4. Mix well and determine the cell density in the algal concentrate. Some cells will be lost in the concentration process.
5. Determine the density of cells (cells/mL) in the stock culture (for this example, assume 2,000,000 per mL).
6. Calculate the required volume of stock culture as follows:

$$\begin{aligned} \text{Volume (mL) of Stock Culture Required} &= \frac{\text{Number test flasks to be used} \times \text{Volume of test Solutions/flask} \times 10,000 \text{ cells/mL}}{\text{Cell density (cells/mL) in the stock culture}} \\ &= \frac{24 \text{ flasks} \times 100 \text{ mL/flask} \times 10,000 \text{ cells/mL}}{2,000,000 \text{ cells/mL}} \\ &= 12.0 \text{ mL Stock Culture} \end{aligned}$$

7. Dilute the cell concentrate as needed to obtain a cell density of 1,000,000 cells/mL, and check the cell density in the final inoculum.
8. The volume of the algal inoculum should be considered in calculating the dilution of toxicant in the test flasks.

14.10.2 START OF THE TEST

14.10.2.1 Label the test chambers with a marking pen and use the color-coded tape to identify each treatment and replicate. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) should have a minimum of four replicates.

14.10.2.2 Randomize the position of the test flasks at the beginning of the test (see Appendix A). Preparation of a position chart may be helpful.

14.10.2.3 The test begins when the algae are added to the test flasks. Mix the inoculum well, and add 1 mL to the test solution in each randomly arranged flask. Make a final check of the cell density in three of the test solutions at time "zero" (within 2 h of the inoculation).

14.10.2.3.1 Alkalinity, hardness, and conductivity are measured at the beginning of the test in the high, medium, and low effluent concentrations and control before they are dispensed to the test chambers and the data recorded on the data sheet (Figure 1).

Discharger: _____ Test Dates: _____
 Location: _____ Analyst: _____

| Effluent Concentration | | | | | | | |
|------------------------|---------|--|--|--|--|--|---------|
| Parameter | Control | | | | | | Remarks |
| Temperature | | | | | | | |
| pH | | | | | | | |
| Alkalinity | | | | | | | |
| Hardness | | | | | | | |
| Conductivity | | | | | | | |
| Chlorine | | | | | | | |

Figure 1. Data form for the green alga, *Selenastrum capricornutum*, growth test. Routine chemical and physical determinations.

14.10.3 LIGHT, PHOTOPERIOD, AND TEMPERATURE

14.10.3.1 Test flasks are incubated under continuous illumination at $86 \pm 8.6 \mu\text{E}/\text{m}^2/\text{s}$ ($400 \pm 40 \text{ ft-c}$), at $25 \pm 1^\circ\text{C}$, and should be shaken continuously at 100 cpm on a mechanical shaker or twice daily by hand. Flask positions in the incubator should be randomly rotated each day to minimize possible spatial differences in illumination and temperature on growth rate. If it can be verified that test specifications are met at all positions, this need not be done.

14.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

14.10.4.1 Because of the continuous illumination of the test flasks, DO concentration should never be a problem during the test and no aeration will be required.

14.10.5 OBSERVATIONS DURING THE TEST

14.10.5.1 Routine Chemical and Physical Determinations

14.10.5.1.1 Temperature should be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be checked in a sufficient number of test vessels at least at the end of the test to determine variability in the environmental chamber.

14.10.5.1.2 Temperature and pH are measured at the end of each 24-h exposure period in at least one test flask at each concentration and in the control.

14.10.5.1.3 Record all the measurements on the data sheet (Figure 1).

14.10.5.2 Biological Observations

14.10.5.2.1 Toxic substances in the test solutions may degrade or volatilize rapidly, and the inhibition in algal growth may be detectable only during the first one or two days in the test. It may be desirable, therefore, to determine the algal growth response daily. Otherwise, biological observations are not required until the test is terminated and the test solutions are not renewed during the test period.

14.10.6 TERMINATION OF THE TEST

14.10.6.1 The test is terminated 96 h after initiation. The algal growth in each flask is measured by one of the following methods: (a) cell counts, (b) chlorophyll content, or (c) turbidity (light absorbance).

14.10.6.2 Cell counts

14.10.6.2.1 Automatic Particle Counters

14.10.6.2.1.1 Several types of automatic electronic and optical particle counters are available for use in the rapid determination of cell density (cells/mL) and mean cell volume (MCV) in $\mu\text{m}^3/\text{cell}$. The Coulter Counter is widely used and is discussed in detail in USEPA (1978b).

14.10.6.2.1.2 If biomass data are desired for algal growth potential measurements, a Model ZM Coulter Counter is used. However, the instrument must be calibrated with a reference sample of particles of known volume.

14.10.6.2.1.3 When the Coulter Counter is used, an aliquot (usually 1 mL) of the test culture is diluted 10X to 20X with a 1% sodium chloride electrolyte solution, such as ISOTON[®], to facilitate counting. The resulting dilution is counted using an aperture tube with a 100- μm diameter aperture. Each cell (particle) passing through the aperture causes a voltage drop proportional to its volume. Depending on the model, the instrument stores the information on the number of particles and the volume of each, and calculates the mean cell volume. The following procedure is used:

1. Mix the algal culture in the flask thoroughly by swirling the contents of the flask approximately six times in a clockwise direction, and then six times in the reverse direction; repeat the two-step process at least once.
2. At the end of the mixing process, stop the motion of the liquid in the flask with a strong brief reverse mixing action, and quickly remove 1 mL of cell culture from the flask with a sterile pipet.
3. Place the aliquot in a counting beaker, and add 9 mL (or 19 mL) of electrolyte solution (such as Coulter ISOTON[®]).
4. Determine the cell density (and MCV, if desired).

14.10.6.2.2 Manual microscope counting method

14.10.6.2.2.1 Cell counts may be determined using a Sedgwick-Rafter, Palmer-Maloney, hemocytometer, inverted microscope, or similar methods. For details on microscope counting methods, see APHA (1992) and USEPA (1973). Whenever feasible, 400 cells per replicate are counted to obtain $\pm 10\%$ precision at the 95% confidence level. This method has the advantage of allowing for the direct examination of the condition of the cells.

14.10.6.3 Chlorophyll Content

14.10.6.3.1 Chlorophyll may be estimated in-vivo fluorometrically, or in-vitro either fluorometrically or spectrophotometrically. In-vivo fluorometric measurements are recommended because of the simplicity and sensitivity of the technique and rapidity with which the measurements can be made (Rehnberg et al., 1982).

14.10.6.3.2 The in-vivo chlorophyll measurements are made as follows:

1. Adjust the "blank" reading of the fluorometer using the filtrate from an equivalent dilution of effluent filtered through a 0.45 μm particle retention filter.
2. Mix the contents of the test culture flask by swirling successively in opposite directions (at least three times), and remove 1 mL of culture from the flask with a sterile pipet.
3. Place the aliquot in a small disposable vial and record the fluorescence as soon as the reading stabilizes. (Do not allow the sample to stand in the instrument more than 1 min).
4. Discard the sample.

14.10.6.3.3 For additional information on chlorophyll measurement methods, (see APHA, 1992).

14.10.6.4 Turbidity (Absorbance)

14.10.6.4.1 A second rapid technique for growth measurement involves the use of a spectrophotometer to determine the turbidity, or absorbance, of the cultures at a wavelength of 750 nm. Because absorbance is a complex function of the volume, size, and pigmentation of the algae, it would be useful to construct a calibration curve to establish the relationship between absorbance and cell density.

14.10.6.4.2 The algal growth measurements are made as follows:

1. A blank is prepared as described for the fluorometric analysis.
2. The culture is thoroughly mixed as described above.
3. Sufficient sample is withdrawn from the test flask with a sterile pipet and transferred to a 1- to 5-cm cuvette.
4. The absorbance is read at 750 nm and divided by the light path length of the cuvette, to obtain an "absorbance-per-centimeter" value.
5. The 1-cm absorbance values are used in the same manner as the cell counts.

14.10.6.5 Record the data as indicated in Figure 2.

14.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

14.11.1 A summary of test conditions and test acceptability criteria is presented in Table 3.

14.12 ACCEPTABILITY OF TEST RESULTS

14.12.1 For the test results to be acceptable, the mean algal cell density in the control flasks must exceed 1×10^6 cells/mL at the end of the test, and the coefficient of variation (CV, calculated as standard deviation $\times 100 /$ mean) for algal cell density among the control replicates must not exceed 20%.

Discharger: _____ Test Dates: _____
 Location: _____ Analyst: _____

| Concentration | Cell Density Measurement | | | | Treatment Mean | Comments |
|---------------|--------------------------|---|---|---|----------------|----------|
| | Replicate | | | | | |
| | 1 | 2 | 3 | 4 | | |
| Control | | | | | | |
| Conc: | | | | | | |
| Conc: | | | | | | |
| Conc: | | | | | | |
| Conc: | | | | | | |

Comments:

Figure 2. Data form for the green alga, *Selenastrum capricornutum*, growth test, cell density determinations.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1003.0)¹

| | | |
|-----|---|---|
| 1. | Test type: | Static non-renewal (required) |
| 2. | Temperature: | 25 ± 1 °C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required) |
| 3. | Light quality: | "Cool white" fluorescent lighting (recommended) |
| 4. | Light intensity: | 86 ± 8.6 µE/m ² /s (400 ± 40 ft-c or 4306 lux) (recommended) |
| 5. | Photoperiod: | Continuous illumination (required) |
| 6. | Test chamber size: | 125 mL or 250 mL (recommended) |
| 7. | Test solution volume: | 50 mL or 100 mL ² (recommended) |
| 8. | Renewal of test solutions: | None (required) |
| 9. | Age of test organisms: | 4 to 7 days (required) |
| 10. | Initial cell density in test chambers: | 10,000 cells/mL (recommended) |
| 11. | No. replicate chambers per concentration: | 4 (required minimum) |
| 12. | Shaking rate: | 100 cpm continuous, or twice daily by hand (recommended) |
| 13. | Aeration: | None (recommended) |
| 14. | Dilution water: | Algal stock culture medium, enriched uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals, or DMW (see Section 7, Dilution Water) (available options) |

¹ For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

² For tests not continuously shaken use 25 mL in 125 mL flasks and 50 mL in 250 mL flasks.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1003.0) (CONTINUED)

| | |
|---|---|
| 15. Test concentrations: | Effluents: 5 and a control (required minimum) Receiving Water: 100% receiving water (or minimum of 5) and a control (recommended) |
| 16. Test dilution factor: | Effluents: ≥ 0.5 (recommended) Receiving Waters: None or ≥ 0.5 (recommended) |
| 17. Test duration: | 96 h (required) |
| 18. Endpoint: | Growth (cell counts, chlorophyll fluorescence, absorbance, or biomass) (required) |
| 19. Test acceptability criteria: ³ | Mean cell density of at least 1×10^6 cells/mL in the controls; and variability (CV%) among control replicates less than or equal to 20% (required) |
| 20. Sampling requirements: | For on-site tests, one sample collected at test initiation, and used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required) |
| 21. Sample volume required: | 1 or 2 L depending on test volume (recommended) |

³ If the test is conducted under non-NPDES applications (i.e., data are not submitted under NPDES permits) and used without EDTA in the nutrient stock solution, the test acceptability criteria are a mean cell density of at least 2×10^5 cells/mL in the controls, and variability (CV%) among control replicates less than or equal to 20%.

14.13 DATA ANALYSIS

14.13.1 GENERAL

14.13.1.1 Tabulate and summarize the data. A sample set of algal growth response data is shown in Table 4.

TABLE 4. GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, GROWTH RESPONSE DATA

| Replicate | Control | Toxicant Concentration ($\mu\text{g Cd/L}$) | | | | | |
|------------------------------------|---------|---|-------|-------|-------|-------|-------|
| | | 5 | 10 | 20 | 40 | 80 | |
| A | 1209 | 1212 | 826 | 493 | 127 | 49.3 | |
| B | 1180 | 1186 | 628 | 416 | 147 | 40.0 | |
| C | 1340 | 1204 | 816 | 413 | 147 | 44.0 | |
| Log ₁₀ Trans- formed | A | 3.082 | 3.084 | 2.917 | 2.693 | 2.104 | 1.693 |
| | B | 3.072 | 3.074 | 2.798 | 2.619 | 2.167 | 1.602 |
| | C | 3.127 | 3.081 | 2.912 | 2.616 | 2.167 | 1.643 |
| Mean(\bar{Y}_i) | | 3.094 | 3.080 | 2.876 | 2.643 | 2.146 | 1.646 |

14.13.1.2 The endpoints of toxicity tests using the green alga, *Selenastrum capricornutum*, are based on the adverse effects on cell growth (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). The EC50, the IC25, and the IC50 are calculated using the point estimation techniques, and LOEC and NOEC values for growth are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the EC50, IC25, and IC50. See the Appendices for examples of the manual computations, and examples of data input and program output.

14.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in Appendix B. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

14.13.2 EXAMPLE OF ANALYSIS OF ALGAL GROWTH DATA

14.13.2.1 Formal statistical analysis of the growth data is outlined on the flowchart in Figure 3. The response used in the statistical analysis is the number of cells per milliliter per replicate. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 and IC50 endpoints.

14.13.2.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the 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 homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Tests, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

STATISTICAL ANALYSIS OF ALGAL GROWTH TEST

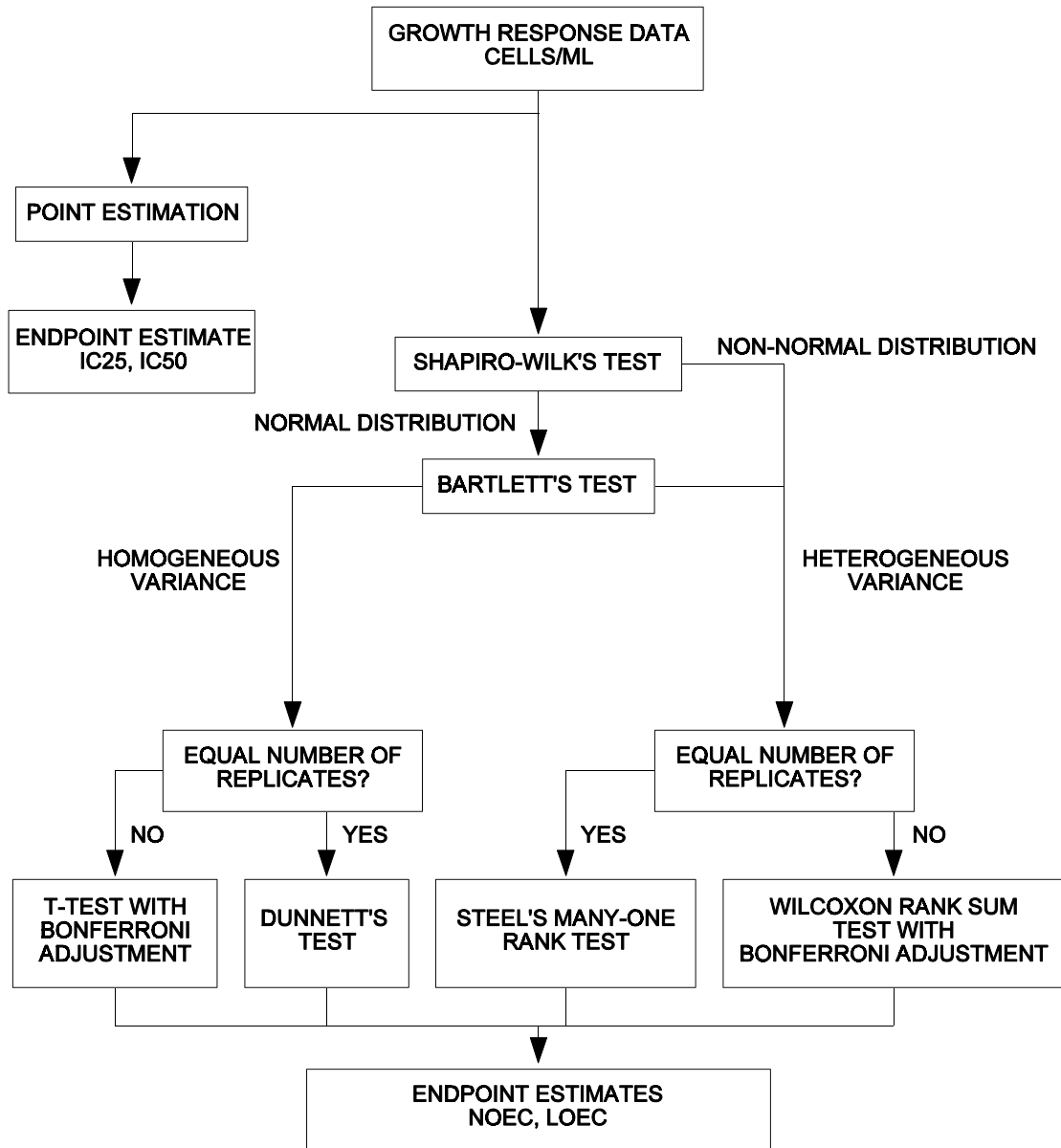


Figure 3. Flowchart for statistical analysis of the green alga, *Selenastrum capricornutum*, growth response data.

14.13.2.3 Additionally, 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 the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

14.13.2.4 Data from an algal growth test with cadmium chloride will be used to illustrate the statistical analysis. The cell counts were \log_{10} transformed in an effort to stabilize the variance for the ANOVA analysis. The raw data, \log_{10} transformed data, mean and standard deviation of the observations at each concentration including the control are listed in Table 4. A plot of the \log_{10} transformed cell counts for each treatment is provided in Figure 4.

14.13.2.5 Test for Normality

14.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 5.

TABLE 5. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

| Replicate | Toxicant Concentration ($\mu\text{g Cd/L}$) | | | | | |
|-----------|---|--------|--------|--------|--------|--------|
| | Control | 5 | 10 | 20 | 40 | 80 |
| A | -0.012 | 0.004 | 0.041 | 0.050 | -0.042 | 0.047 |
| B | -0.022 | -0.006 | -0.078 | -0.024 | 0.021 | -0.044 |
| C | 0.033 | 0.001 | 0.036 | -0.027 | 0.021 | -0.003 |

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

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

Where: X_i = the i th centered observation

\bar{X} = the overall mean of the centered observations

n = the total number of centered observations.

For this set of data, $n = 18$

$$\bar{X} = \frac{1}{18}(0.000) = 0.000$$

$$D = 0.0214$$

14.13.2.5.3 Order the centered observations from smallest to largest:

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

Where $X^{(i)}$ is the i th ordered observation. These ordered observations are listed in Table 6.

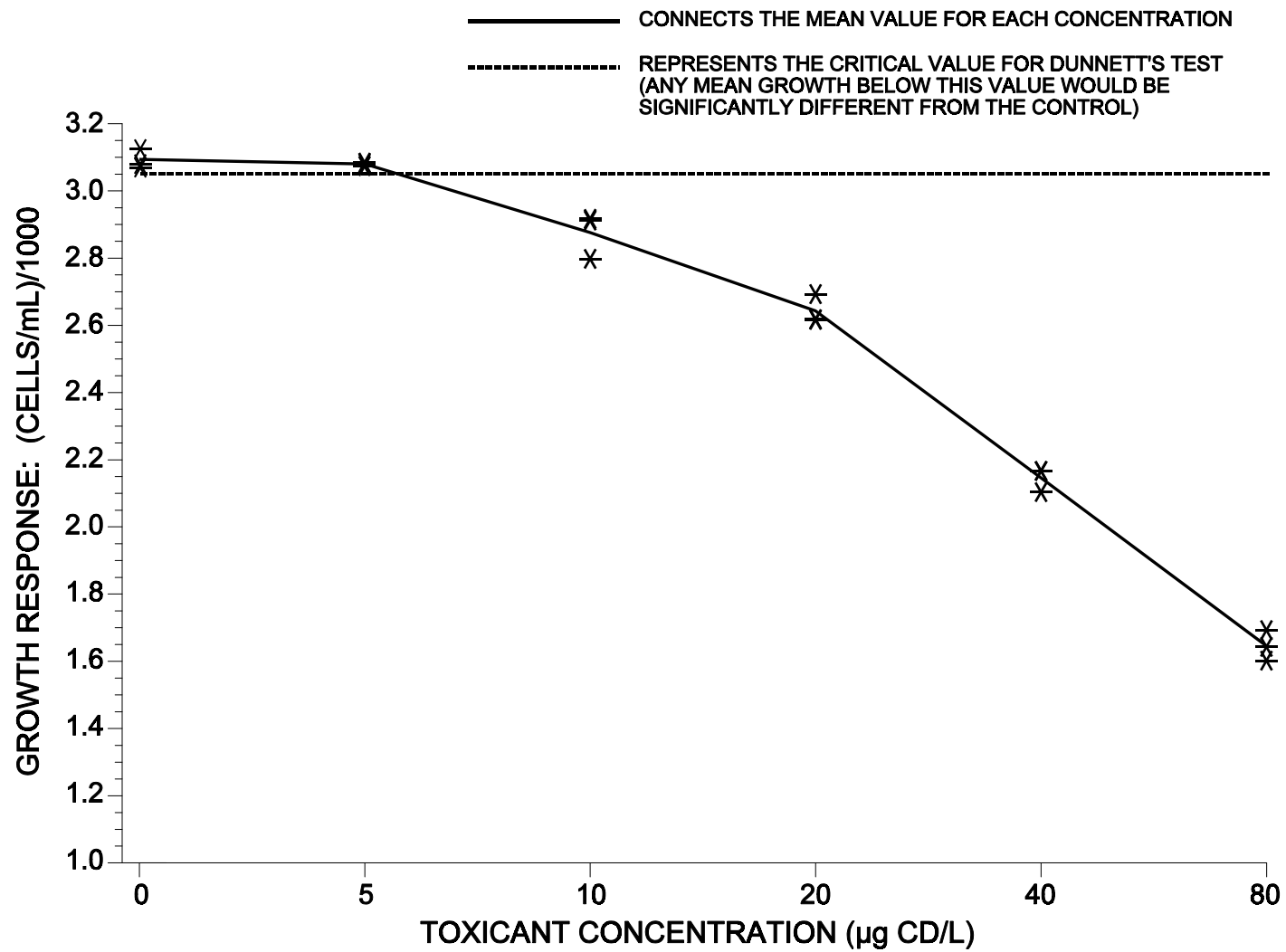


Figure 4. Plot of the \log_{10} transformed cell count data from the green alga, *Selenastrum capricornutum*, growth response test in Table 4.

TABLE 6. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

| i | $X^{(i)}$ | i | $X^{(i)}$ |
|---|-----------|----|-----------|
| 1 | -0.078 | 10 | 0.001 |
| 2 | -0.044 | 11 | 0.004 |
| 3 | -0.042 | 12 | 0.021 |
| 4 | -0.027 | 13 | 0.021 |
| 5 | -0.024 | 14 | 0.033 |
| 6 | -0.022 | 15 | 0.036 |
| 7 | -0.012 | 16 | 0.041 |
| 8 | -0.006 | 17 | 0.047 |
| 9 | -0.003 | 18 | 0.050 |

14.13.2.5.4 From Table 4, Appendix B, for the number of observations, n , obtain the coefficients a_1, a_2, \dots, a_k where k is $n/2$ if n is even and $(n-1)/2$ if n is odd. For the data in this example, $n = 18$, $k = 9$. The a_i values are listed in Table 7.

14.13.2.5.5 Compute the test statistic, W , as follows:

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

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 7.

TABLE 7. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

| i | a_i | $X^{(n-i+1)} - X^{(i)}$ | |
|---|--------|-------------------------|----------------------|
| 1 | 0.4886 | 0.128 | $X^{(18)} - X^{(1)}$ |
| 2 | 0.3253 | 0.091 | $X^{(17)} - X^{(2)}$ |
| 3 | 0.2553 | 0.083 | $X^{(16)} - X^{(3)}$ |
| 4 | 0.2027 | 0.063 | $X^{(15)} - X^{(4)}$ |
| 5 | 0.1587 | 0.057 | $X^{(14)} - X^{(5)}$ |
| 6 | 0.1197 | 0.043 | $X^{(13)} - X^{(6)}$ |
| 7 | 0.0837 | 0.033 | $X^{(12)} - X^{(7)}$ |
| 8 | 0.0496 | 0.010 | $X^{(11)} - X^{(8)}$ |
| 9 | 0.0163 | 0.004 | $X^{(10)} - X^{(9)}$ |

For this set of data:

$$W = \frac{1}{0.0214}(0.1436)^2 = 0.964$$

14.13.2.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 18 observations (n) is 0.858. Since $W = 0.964$ is greater than the critical value, the conclusion of the test is that the data are normally distributed.

14.13.2.6 Test for Homogeneity of Variance

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

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

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

p = number of levels of toxicant concentration including the control

n_i = the number of replicates for concentration i

$\ln = \log_e$

$i = 1, 2, \dots, p$, where p is the number of concentrations including the control

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

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

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

14.13.2.6.3 Bartlett's statistic is therefore:

$$\begin{aligned} B &= \frac{[(12) \ln(0.0018) - 2 \sum_{i=1}^P \ln(S_i^2)]}{1.194} \\ &= [12(-6.3200) - 2(-41.9082)]/1.194 \\ &= 7.9764/1.194 \\ &= 6.6804 \end{aligned}$$

14.13.2.6.4 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 15.09. Since B = 6.6804 is less than the critical value of 15.09, conclude that the variances are not different.

14.13.2.7 Dunnett's Procedure

14.13.2.7.1 To obtain an estimate of the pooled variance for Dunnett's Procedure, construct an ANOVA table as described in Table 8.

TABLE 8. ANOVA TABLE

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

Where: p = number of toxicant concentrations including the control

N = total number of observations $n_1 + n_2 \dots + n_p$

n_i = number of observations in concentration i

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

$$SST = \sum_{i=1}^p \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N} \quad \text{Total Sum of Squares}$$

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

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

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the cell count for toxicant concentration i in test chamber j)

14.13.2.7.2 For the data in this example:

$$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 3$$

$$N = 18$$

$$T_1 = Y_{11} + Y_{12} + Y_{13} = 9.281$$

$$T_2 = Y_{21} + Y_{22} + Y_{23} = 9.239$$

$$T_3 = Y_{31} + Y_{32} + Y_{33} = 8.627$$

$$T_4 = Y_{41} + Y_{42} + Y_{43} = 7.928$$

$$T_5 = Y_{51} + Y_{52} + Y_{53} = 6.438$$

$$T_6 = Y_{61} + Y_{62} + Y_{63} = 4.938$$

$$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 46.451$$

$$\begin{aligned} SSB &= \sum_{i=1}^p \frac{T_i^2}{n_i} - \frac{G^2}{N} \\ &= \frac{1}{3}(374.606) - \frac{(46.451)^2}{18} = 4.997 \end{aligned}$$

$$\begin{aligned} SST &= \sum_{i=1}^p \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N} \\ &= 124.890 - \frac{(46.451)^2}{18} = 5.018 \end{aligned}$$

$$SSW = SST - SSB = 5.018 - 4.997 = 0.0210$$

$$S_B^2 = SSB/(p-1) = 4.996/(6-1) = 0.9990$$

$$S_W^2 = SSW/(N-p) = 0.021/(18-6) = 0.0018$$

14.13.2.7.3 Summarize these calculations in the ANOVA table (Table 9).

TABLE 9. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

| Source | df | Sum of Squares (SS) | Mean Square (MS) (SS/df) |
|---------|----|---------------------|--------------------------|
| Between | 5 | 4.997 | 0.999 |
| Within | 12 | 0.021 | 0.0018 |
| Total | 17 | 5.017 | |

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

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

Where: \bar{Y}_i = mean cell count for toxicant concentration i

\bar{Y}_1 = mean cell count 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.

14.13.2.7.5 Table 10 includes the calculated t values for each concentration and control combination. In this example, comparing the 5 $\mu\text{g/L}$ concentration with the control the calculation is as follows:

$$t_2 = \frac{(3.094 - 3.080)}{[0.0424\sqrt{(1/3) + (1/3)}]} = 0.405$$

TABLE 10. CALCULATED T VALUES

| Toxicant Concentration ($\mu\text{g Cd/L}$) | i | t_i |
|---|---|--------|
| 5 | 2 | 0.405 |
| 10 | 3 | 6.300 |
| 20 | 4 | 13.035 |
| 40 | 5 | 27.399 |
| 80 | 6 | 41.850 |

14.13.2.7.6 Since the purpose of this test is to detect a significant reduction in mean cell count, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 12 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.50. The mean count for concentration i is considered significantly less than the mean count for the control if t_i is greater than the critical value. Since t_3 , t_4 , t_5 and t_6 are greater than 2.50, the 10, 20, 40 and 80 $\mu\text{g/L}$ concentrations have significantly lower mean cell counts than the control. Hence the NOEC and the LOEC for the test are 5 $\mu\text{g/L}$ and 10 $\mu\text{g/L}$, respectively.

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

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

Where: d = the critical value for 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_1 = the number of replicates in the control.

14.13.2.7.8 In this example:

$$\begin{aligned} MSD &= 2.50(0.0424)\sqrt{(1/3)+(1/3)} \\ &= 2.50 (0.0424)(0.8165) \\ &= 0.086 \end{aligned}$$

14.13.2.7.9 The MSD (0.086) is in transformed units. An approximate MSD in terms of cell count per 100 mL may be calculated via the following conversion.

1. Subtract the MSD from the transformed control mean.

$$3.094 - 0.086 = 3.008$$

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

$$10^{(3.094)} = 1241.6$$

$$10^{(3.008)} = 1018.6$$

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

$$MSU_u = 1241.6 - 1018.6 = 223$$

14.13.2.7.10 Therefore, for this set of data, the minimum difference in mean cell count between the control and any toxicant concentration that can be detected as statistically significant is 223.

14.13.2.7.11 This represents a decrease in growth of 18% from the control.

14.13.2.8 Calculation of the IC_p

14.13.2.8.1 The growth data in Table 4 are utilized in this example. Table 11 contains the means for each toxicant concentration. As can be seen, the observed means are monotonically non-increasing with respect to concentration. Therefore, it is not necessary to smooth the means prior to calculating the IC_p. See Figure 5 for a plot of the response curve.

TABLE 11. ALGAL MEAN GROWTH RESPONSE AFTER SMOOTHING

| Toxicant Concentration ($\mu\text{g Cd/L}$) | i | Response means, \bar{Y}_i (cells/mL) | Smoothed mean, M_i (cells/mL) |
|--|---|--|---------------------------------------|
| Control | 1 | 1243 | 1243 |
| 5 | 2 | 1201 | 1201 |
| 10 | 3 | 757 | 757 |
| 20 | 4 | 441 | 441 |
| 40 | 5 | 140 | 140 |
| 80 | 6 | 44 | 44 |

14.13.2.8.2 An IC₂₅ and IC₅₀ can be estimated using the Linear Interpolation Method (Appendix M). A 25% reduction in cell count, compared to the controls, would result in a mean count of 932 cells, where $M_1(1-p/100) = 1243(1-25/100)$. A 50% reduction in cell count, compared to the controls, would result in a mean count of 622 cells. Examining the means and their associated concentrations (Table 11), the response, 932 cells, is bracketed by $C_2 = 5 \mu\text{g Cd/L}$ and $C_3 = 10 \mu\text{g Cd/L}$. The response, 622 cells, is bracketed by $C_3 = 10 \mu\text{g Cd/L}$ and $C_4 = 20 \mu\text{g Cd/L}$.

14.13.2.8.3 Using the equation from section 4.2 of Appendix M, the estimate of the IC₂₅ is calculated as follows:

$$IC_p = C_j + [M_1(1-p/100) - M_j] \frac{(C_{j+1} - C_j)}{(M_{j+1} - M_j)}$$

$$IC_{25} = 5 + [1243(1-25/100) - 1201] \frac{(10-5)}{(757-1201)}$$

$$= 8 \mu\text{g Cd/L.}$$

14.13.2.8.4 The IC₅₀ estimate is $14 \mu\text{g Cd/L}$:

$$IC_{25} = 6.25 + [28.75(1-25/100) - 28.75] \frac{(12.5-6.25)}{(9.40-28.75)}$$

$$IC_{50} = 10 + [1243(1-50/100) - 757] \frac{(20-10)}{(441-757)}$$

$$= 14 \mu\text{g Cd/L.}$$

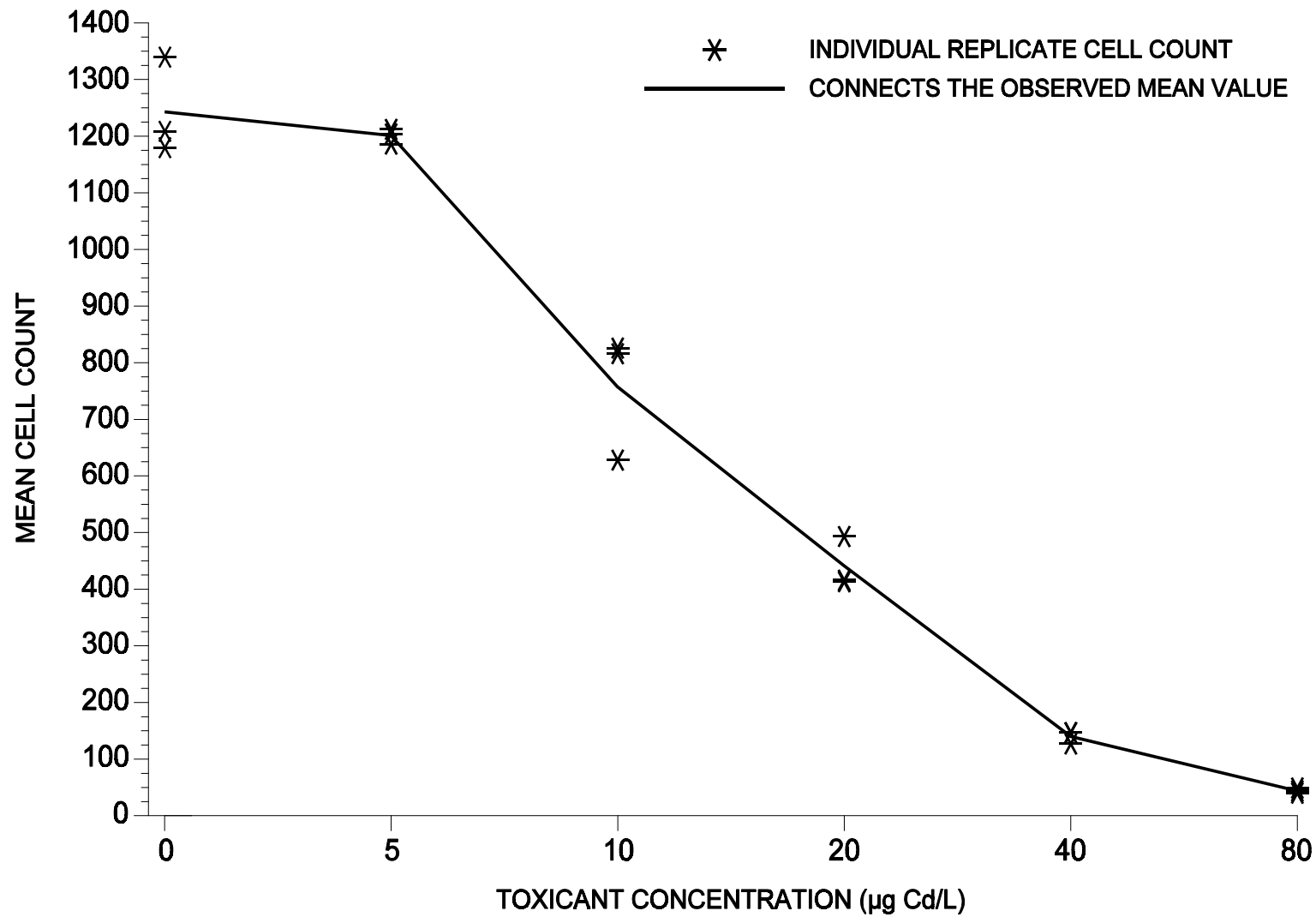


Figure 5. Plot of raw data and observed means for the green alga, *Selenastrum capricornutum*, growth data.

14.13.2.8.5 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 8.0227 µg Cd/L. The empirical 95% confidence interval for the true mean was 6.4087 µg Cd/L and 10.0313 µg Cd/L. The ICPIN computer program output for the IC25 for this data set is shown in Figure 6.

14.13.2.8.6 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 14.2774 µg Cd/L. The empirical 95% confidence interval for the true mean was 9.7456 µg Cd/L and 18.5413 µg Cd/L. The computer program output for the IC50 for this data set is shown in Figure 7.

14.13.3 BIOSTIMULATION

14.13.3.1 Where the growth response in effluent (or surface water) exceeds growth in the control flasks, the percent stimulation, S(%), is calculated as shown below. Values which are significantly greater than the control indicate a possible degrading enrichment effect on the receiving water (Walsh et al., 1980):

$$S (\%) = \frac{T - C}{C} \times 100$$

Where: T = Mean effluent or surface water response

C = Mean control response

14.14 PRECISION AND ACCURACY

14.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 14.14.1.1 and 14.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

14.14.1.1 Single-Laboratory Precision

14.14.1.1.1 Data from repetitive 96-h toxicity tests conducted with cadmium chloride as the reference toxicant, using medium containing EDTA, are shown in Table 12. The precision (CV) of the 10 EC50s was 10.2%.

14.14.1.1.2 EPA evaluated within-laboratory precision of the green alga, *Selenastrum capricornutum*, Growth Test using a database of routine reference toxicant test results from nine laboratories (USEPA, 2000b). The database consisted of 85 reference toxicant tests conducted in 9 laboratories using a variety of reference toxicants including: copper, sodium chloride, and zinc. Among the 9 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 26% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 25%; and in 75% of laboratories, the within-laboratory CV was less than 39%.

14.14.1.2 Multilaboratory Precision

14.14.1.2.1 In 2000, EPA conducted an interlaboratory variability study of the green alga, *Selenastrum capricornutum*, Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 11 participant laboratories tested 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of moderately-hard synthetic freshwater, the effluent sample was a

municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Each sample was tested with and without the addition of EDTA. Of the 44 *Selenastrum capricornutum* Growth tests conducted with EDTA, 63.6% were successfully completed and met the required test acceptability criteria. Of the 44 tests conducted without EDTA, 65.9% were successfully completed and met the required test acceptability criteria. Of five tests that were conducted on blank samples with the addition of EDTA, none showed false positive results for the growth endpoint. Of 6 tests that were conducted on blank samples without the addition of EDTA, 2 showed false positive results for the growth endpoint, yielding a false positive rate of 33.3%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 13 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 34.3% and 58.5% for IC25 results in tests with EDTA and without EDTA, respectively. Table 14 shows the precision of growth NOEC endpoints for each sample type. NOEC values for tests with EDTA spanned three concentrations for the effluent sample type and four concentrations for the reference toxicant and receiving water sample types. NOEC values for tests without EDTA, spanned six concentrations for the reference toxicant sample type, four concentrations for the effluent sample type, and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median for tests conducted with EDTA was 85.7%, 100%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively. The percentage of values within one concentration of the median for tests conducted without EDTA was 40.0%, 50.0%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

14.14.2 ACCURACY

14.14.2.1 The accuracy of toxicity tests cannot be determined.

| Conc. ID | 1 | 2 | 3 | 4 | 5 | 6 |
|--------------|------|------|-----|-----|-----|------|
| Conc. Tested | 0 | 5 | 10 | 20 | 40 | 80 |
| Response 1 | 1209 | 1212 | 826 | 493 | 127 | 49.3 |
| Response 2 | 1180 | 1186 | 628 | 416 | 147 | 40.0 |
| Response 3 | 1340 | 1204 | 816 | 413 | 147 | 44.0 |

*** Inhibition Concentration Percentage Estimate ***

Toxicant/Effluent: Cadmium
 Test Start Date: Example Test Ending Date:
 Test Species: Selenastrum capricornutum
 Test Duration: 96 h
 DATA FILE: scmanual.icp
 OUTPUT FILE: scmanual.i25

| Conc. ID | Number Replicates | Concentration µg/l | Response Means | Std. Dev. | Pooled Response Means |
|----------|-------------------|--------------------|----------------|-----------|-----------------------|
| 1 | 3 | 0.000 | 1243.000 | 85.247 | 1243.000 |
| 2 | 3 | 5.000 | 1200.667 | 13.317 | 1200.667 |
| 3 | 3 | 10.000 | 756.667 | 111.541 | 756.667 |
| 4 | 3 | 20.000 | 440.667 | 45.347 | 440.667 |
| 5 | 3 | 40.000 | 140.333 | 11.547 | 140.333 |
| 6 | 3 | 80.000 | 44.433 | 4.665 | 44.433 |

The Linear Interpolation Estimate: 8.0227 Entered P Value: 25

Number of Resamplings: 80
 The Bootstrap Estimates Mean: 8.1627 Standard Deviation: 0.4733
 Original Confidence Limits: Lower: 7.2541 Upper: 8.9792
 Expanded Confidence Limits: Lower: 6.4087 Upper: 10.0313
 Resampling time in Seconds: 1.65 Random Seed: -1575623987

Figure 6. ICPIN program output for the IC25.

| Conc. ID | 1 | 2 | 3 | 4 | 5 | 6 |
|--------------|------|------|-----|-----|-----|------|
| Conc. Tested | 0 | 5 | 10 | 20 | 40 | 80 |
| Response 1 | 1209 | 1212 | 826 | 493 | 127 | 49.3 |
| Response 2 | 1180 | 1186 | 628 | 416 | 147 | 40.0 |
| Response 3 | 1340 | 1204 | 816 | 413 | 147 | 44.0 |

*** Inhibition Concentration Percentage Estimate ***

Toxicant/Effluent: Cadmium
 Test Start Date: Example Test Ending Date:
 Test Species: Selenastrum capricornutum
 Test Duration: 96 h
 DATA FILE: scmanual.icp
 OUTPUT FILE: scmanual.i50

| Conc. ID | Number Replicates | Concentration $\mu\text{g/l}$ | Response Means | Std. Dev. | Pooled Response Means |
|----------|-------------------|-------------------------------|----------------|-----------|-----------------------|
| 1 | 3 | 0.000 | 1243.000 | 85.247 | 1243.000 |
| 2 | 3 | 5.000 | 1200.667 | 13.317 | 1200.667 |
| 3 | 3 | 10.000 | 756.667 | 111.541 | 756.667 |
| 4 | 3 | 20.000 | 440.667 | 45.347 | 440.667 |
| 5 | 3 | 40.000 | 140.333 | 11.547 | 140.333 |
| 6 | 3 | 80.000 | 44.433 | 4.665 | 44.433 |

The Linear Interpolation Estimate: 14.2774 Entered P Value: 50

Number of Resamplings: 80
 The Bootstrap Estimates Mean: 14.2057 Standard Deviation: 1.1926
 Original Confidence Limits: Lower: 12.1194 Upper: 16.3078
 Expanded Confidence Limits: Lower: 9.7456 Upper: 18.5413
 Resampling time in Seconds: 1.65 Random Seed: -1751550803

Figure 7. ICPIN program output for the IC50.

TABLE 12. SINGLE LABORATORY PRECISION OF THE GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, 96-H TOXICITY TESTS, USING THE REFERENCE TOXICANT CADMIUM CHLORIDE (USEPA, 1991a)

| Test Number | EC ₅₀ (mg/L) |
|-------------|-------------------------|
| 1 | 2.3 |
| 2 | 2.4 |
| 3 | 2.3 |
| 4 | 2.8 |
| 5 | 2.6 |
| 6 | 2.1 |
| 7 | 2.1 |
| 8 | 2.1 |
| 9 | 2.6 |
| 10 | 2.4 |
| n | 10.0 |
| Mean | 2.37 |
| CV (%) | 10.2 |

TABLE 13. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES¹

| Test Endpoint | Sample Type | CV (%) ² | | |
|------------------------|--------------------|-------------------------|--------------------------|--------------------|
| | | Within-lab ³ | Between-lab ⁴ | Total ⁵ |
| IC25 (with EDTA) | Reference toxicant | 10.9 | 20.8 | 23.5 |
| | Effluent | 39.5 | 8.48 | 40.4 |
| | Receiving water | - | - | 38.9 |
| Average | | 25.2 | 14.6 | 34.3 |
| IC25 (without EDTA) | Reference toxicant | 25.6 | 83.6 | 87.5 |
| | Effluent | 21.0 | 60.3 | 63.9 |
| | Receiving water | - | - | 24.1 |
| Average | | 23.3 | 72.0 | 58.5 |

¹ From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

² CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

³ The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

⁴ The between-laboratory component of variability for duplicate samples tested at different laboratories.

⁵ The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 14. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES¹

| Test Endpoint | Sample Type | Median NOEC Value | % of Results at the Median | % of Results ± 1 ² | % of Results ≥ 2 ³ |
|-------------------------------|--------------------|-------------------|----------------------------|-----------------------------------|------------------------------------|
| Growth NOEC (with EDTA) | Reference toxicant | 25% | 57.1 | 28.6 | 14.3 |
| | Effluent | 6.25% | 42.9 | 57.1 | 0.00 |
| | Receiving water | 12.5% | 28.6 | 57.1 | 14.3 |
| Growth NOEC (without EDTA) | Reference toxicant | 18.8% | - ⁴ | 40.0 | 60.0 |
| | Effluent | 18.8% | - ⁴ | 50.0 | 50.0 |
| | Receiving water | 6.25% | 75.0 | 25.0 | 0.00 |

¹ From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

² Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.

³ Percent of values two or more concentration intervals above or below the median.

⁴ The median NOEC fell between test concentrations, so no test results fell precisely on the median.