

EG-8
August, 1982

ALGAL ACUTE TOXICITY TEST

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(a) Purpose. The guideline in this section is intended for use in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (P.L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 et seq.). This guideline prescribes test procedures and conditions using freshwater and marine algae to develop data on the phytotoxicity of chemicals. The United States Environmental Protection Agency (USEPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) Definitions. The definitions in Section 3 of the Toxic Substances Control Act (TSCA) and the definitions in Part 792-- Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this guideline:

- (1) "Algicidal" means having the property of killing algae.
- (2) "Algistatic" means having the property of inhibiting algal growth.
- (3) "ECx" means the experimentally derived chemical concentration that is calculated to effect X percent of the test

criterion.

(4) "Growth" means a relative measure of the viability of an algal population based on the number and/or weight of algal cells per volume of nutrient medium or test solution in a specified period of time.

(5) "Static system" means a test container in which the test solution is not renewed during the period of the test.

(c) Test procedures--(1) Summary of the test. (A) In preparation for the test, fill test containers with appropriate volumes of nutrient medium and/or test solution. Start the test by introducing algae into the test and control containers in the growth chambers. Environmental conditions within the growth chambers are established at predetermined limits.

(B) At the end of 96 hours enumerate the algal cells in all containers to determine inhibition or stimulation of growth in test containers compared to controls. Use data to define the concentration-response curve, and calculate the EC-10, EC-50, and EC-90 values.

(2) [Reserved]

(3) Range-finding test. (i) A range-finding test should be conducted to determine if:

(A) definitive testing is necessary

(B) test chemical concentrations for the definitive test.

(ii) Algae are exposed to a widely spaced (e.g., log interval) chemical concentration series. The lowest value in the series, exclusive of controls, should be at the chemical's detection limit. The upper value, for water soluble compounds, should be the saturation concentration. No replicates are required; and nominal concentrations of the chemical are acceptable unless definitive testing is not required.

(iii) The test is performed once for each of the recommended algal species or selected alternates. Test chambers should contain equal volumes of test solution and approximately 1×10^4 Selenastrum cells/ml or 7.7×10^4 Skeletonema cells/ml of test solution. The algae should be exposed to each concentration of test chemical for up to 96 hours. The exposure period may be shortened if data suitable for the purposes of the range-finding test can be obtained in less time.

(iv) Definitive testing is not necessary if the highest chemical concentration tested (water saturation concentration or 1000 mg/l) results in less than a 50 percent reduction in growth or if the lowest concentration tested (analytical detection limit) results in greater than a 50 percent reduction in growth.

(4) Definitive test. (i) The purpose of the definitive test is to determine the concentration response curves, the EC-10's, EC-50's, and EC-90's for algal growth for each species

tested, with a minimum amount of testing beyond the range-finding test.

(ii) Algae should be exposed to five or more concentrations of the test chemical in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 6, 8, 16, 32 and 64 mg/l). Algae should be placed in a minimum of three replicate test containers for each concentration of test chemical and control. More than three replicates may be required to provide sufficient quantities of test solution for determination of test substance concentration at the end of the test. Each test chamber should contain equal volumes of test solution and approximately 1×10^4 Selenastrum cells ml^{-1} or 7.7×10^4 Skeletonema cells/ml of test solution. The chemical concentrations should result in greater than 90 percent of algal growth being inhibited or stimulated at the lowest concentrations of test substance compared to controls.

(iii) Every test should include a control consisting of the same nutrient medium, conditions, procedures, and algae from the same culture, except that none of the test substance is added. If a carrier is present in any of the test chambers, a separate carrier control is required.

(iv) The test begins when algae from seven to ten-day-old stock cultures are placed in the test chambers containing test solutions having the appropriate concentrations of the test

substance. Algal growth in controls should reach the logarithmic growth phase by 96 hours (at which time the number of algal cells should be approximately 1.5×10^6 /ml for Skeletonema or 3.5×10^6 /ml for Selenastrum). If growth in controls does not reach this logarithmic phase within this 96-hour period, the test is invalidated and should be repeated. At the end of 96 hours the algal growth response (number or weight of algal cells/ml) in all test containers and controls should be determined by an indirect (spectrophotometry, electronic cell counters, dry weight, etc.) or a direct (actual microscopic cell count) method. Indirect methods should be calibrated by a direct microscopic count. The percentage inhibition or stimulation of growth for each concentration, EC-10, EC-50, EC-90 and the concentration-response curves are determined from these counts.

(v) At the end of the definitive test, the following additional analyses of algal growth response should be performed:

(1) Determine whether the altered growth response between controls and test algae was due to a change in relative cell numbers, cell sizes or both. Also note any unusual cell shapes, color differences, flocculations, adherence of algae to test containers, or aggregation of algal cells.

(2) In test concentrations where growth is maximally inhibited, algistatic effects may be differentiated from

algicidal effects by the following two methods:

(A) Add 0.5 ml of a 0.1 percent solution (weight/volume) of Evans blue stain to a one milliliter aliquot of algae from a control container and to a one milliliter aliquot of algae from the test container having the lowest concentration of test chemical which completely inhibited algal growth (if algal growth was not completely inhibited, select an aliquot of algae for staining from the test container having the highest concentration of test chemical which inhibited algal growth). Wait ten to thirty minutes, examine microscopically, and determine the percent of the cells which stain blue (indicating cell mortality). A staining control should be performed concurrently using heat-killed or formaldehyde-preserved algal cells; 100 percent of these cells should stain blue.

(B) Remove 0.5 ml aliquots of test solution containing growth-inhibited algae from each replicate test container having the concentration of test substance evaluated in (2)(I) above. Combine these aliquots into a new test container and add a sufficient volume of fresh nutrient medium to dilute the test chemical to a concentration which does not affect growth. Incubate this subculture under the environmental conditions used in the definitive test for a period of up to nine days, and observe for algal growth to determine if the algistatic effect

noted after the 96-hour test is reversible. This subculture test may be discontinued as soon as growth occurs.

(5) [Reserved]

(6) Analytical measurements--(i) Chemical. (A) Glass distilled or deionized water should be used in the preparation of the nutrient medium. The pH of the test solution should be measured in the control and test containers at the beginning and at the end of the definitive test. The concentration of test chemical in the test containers should be determined at the beginning and end of the definitive test by standard analytical methods which have been validated prior to the test. An analytical method is unacceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference.

(B) At the end of the test and after aliquots have been removed for algal growth-response determinations, microscopic examination, mortal staining, or subculturing, the replicate test containers for each chemical concentration may be pooled into one sample. An aliquot of the pooled sample may then be taken and the concentration of test chemical determined. In addition, the concentration of test chemical associated with the algae alone should be determined. Separate and concentrate the algal cells from the test solution by centrifuging or filtering the remaining

pooled sample and measure the test substance concentration in the algal-cell concentrate.

(ii) Numerical. Algal growth response (as percent of inhibition or stimulation in the test solutions compared to the controls) is calculated at the end of the test. Mean and standard deviation should be calculated and plotted for each treatment and control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration response curves. The concentration response curves are plotted using the mean measured test solution concentrations obtained at the end of the test.

(d) Test conditions--(1) Test species. Species of algae recommended as test organisms for this test are the freshwater green alga, Selenastrum capricornutum, and the marine diatom, Skeletonema costatum. Algae to be used in acute toxicity tests may be initially obtained from commercial sources and subsequently cultured using sterile technique. Toxicity testing should not be performed until algal cultures are shown to be actively growing (i.e. capable of logarithmic growth within the test period) in at least two subcultures lasting seven days each prior to the start of the definitive test. All algae used for a particular test should be from the same source and the same stock culture. Test algae should not have been used in a previous

test, either in a treatment or a control.

(2) Facilities--(i) General. (A) Facilities needed to perform this test includes: a growth chamber or a controlled environment room that can hold the test containers and will maintain the air temperature, lighting intensity and photoperiod specified in this test guideline; apparatus for culturing and enumerating algae; a source of distilled and/or deionized water; and apparatus for carrying out analyses of the test chemical.

(B) Disposal facilities should be adequate to accommodate spent glassware, algae and test solutions at the end of the test and any bench covering, lab clothing, or other contaminated materials.

(ii) Test containers. Erlenmeyer flasks should be used for test containers. The flasks may be of any volume between 125 and 500 ml as long as the same size is used throughout a test and the test solution volume does not exceed 50 percent of the flask volume.

(iii) Cleaning and sterilization. New test containers may contain substances which inhibit growth of algae. They should therefore be cleaned thoroughly and used several times to culture algae before being used in toxicity testing. All glassware used in algal culturing or testing should be cleaned and sterilized prior to use according to standard good laboratory practices.

(iv) Conditioning. Test containers should be conditioned by a rinse with the appropriate test solutions prior to the start of the test. Decant and add fresh test solutions after an appropriate conditioning period for the test chemical.

(v) Nutrient medium. (A) Formulation and sterilization of nutrient medium used for algal culture and preparation of test solutions should conform to those currently recommended by the U.S. EPA for freshwater and marine algal bioassays. No chelating agents should be included in the nutrient medium used for test solution preparation. Nutrient medium should be freshly prepared for algal testing, and may be dispensed in appropriate volumes in test containers and sterilized by autoclaving or filtration. The pH of the nutrient medium should be 7.5 for Selenastrum and 8.1 for Skeletonema at the start of the test and may be adjusted prior to test chemical addition with 0.1N NaOH or HCl.

(B) Dilution water used for preparation of nutrient medium and test solutions should be filtered, deionized or glass distilled. Saltwater for marine algal nutrient medium and test solutions should be prepared by adding a commercial, synthetic, sea salt formulation or a modified synthetic seawater formulation to distilled/deionized water to a concentration of 30 parts per thousand.

(vi) Carriers. Nutrient medium should be used in making

stock solutions of the test chemical. If a carrier other than nutrient medium is absolutely necessary to dissolve the chemical, the volume used should not exceed the minimum volume necessary to dissolve or suspend the chemical in the test solution.

(3) Test parameters. (A) The test temperature should be maintained at $24^{\circ}\pm 1^{\circ}\text{C}$ for Selenastrum and $20^{\circ}\pm 1^{\circ}\text{C}$ for Skeletonema. Temperature should be recorded hourly during the test.

(B) Test chambers containing Selenastrum should be illuminated continuously and those containing Skeletonema should be provided a 14-hour light and 10-hour dark photoperiod with a 30 minute transition period under fluorescent lamps providing $300 \pm 25 \text{ uEin/m}^2 \text{ sec}$ (approximately 400 ft-c) measured adjacent to the test chambers at the level of test solution.

(C) Stock algal cultures should be shaken twice daily by hand. Test containers should be placed on a rotary shaking apparatus and oscillated at approximately 100 cycles/min for Selenastrum and at approximately 60 cycles/min for Skeletonema during the test. The rate of oscillation should be determined at least once daily during testing.

(D) The pH of nutrient medium in which algae are subcultured should be 7.5 for Selenastrum and 8.1 for Skeletonema, and is not adjusted after the addition of the algae. The pH of all test

solutions and controls should be measured at the beginning and end of the test.

(E) Light intensity should be monitored at least daily during the test at the level of the test solution.

(e) Reporting. The sponsor should submit to the EPA all data developed by the test that are suggestive or predictive of acute phytotoxicity. In addition to the general reporting requirements prescribed in Part 792--Good Laboratory Practice Standards, the following should be reported:

(i) Detailed information about the test organisms, including the scientific name, method of verification, and source;

(ii) A description of the test chambers and containers, the volumes of solution in the containers, the way the test was begun (e.g. conditioning, test substance additions, etc.), the number of replicates, the temperature, the lighting, and method of incubation, oscillation rates, and type of apparatus;

(iii) The concentration of the test chemical in the control and in each treatment at the end of the test and the pH of the solutions;

(iv) The number of algal cells in each treatment and control and the method used to derive these values at the beginning and end of the test; the percentage of inhibition or stimulation of

growth relative to controls; and other adverse effect in the control and in each treatment;

(v) The 96-hour EC-10, EC-50 and EC-90 values and their 95-percent confidence limits, the methods used to derive these values, the data used to define the shape of the concentration-response curve and the goodness-of-fit determination;

(vi) Methods and data records of all chemical analyses of water quality and test substance concentrations, including method validations and reagent blanks;

(vii) The results of any optional analyses such as: microscopic appearance of algae, size or color changes, percent mortality of cells and the fate of subcultured cells, the concentration of test substance associated with algae and test solution supernate or filtrate;

(viii) If the range-finding test showed that the highest concentration of the chemical tested (not less than 1000 mg/l or saturation concentration) had no effect on the algae, report the results and concentration and a statement that the chemical is of minimum phytotoxic concern;

(ix) If the range-finding test showed greater than a 50 percent inhibition of algal growth at a test concentration below the analytical detection limit, report the results, concentration, and a statement that the chemical is phytotoxic below the analytical detection limit.