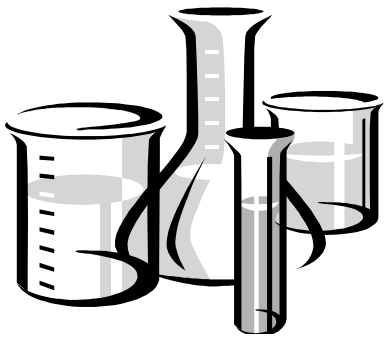
United States
Environmental Protection
Agency

Office of Chemical Safety
and Pollution Prevention
(7101)

EPA 712-C-008
January 2012

Ecological Effects Test Guidelines

OCSPP 850.4400: Aquatic Plant Toxicity Test Using *Lemna* spp.



NOTICE

This guideline is one of a series of test guidelines established by the United States Environmental Protection Agency's Office of Chemical Safety and Pollution Prevention (OCSPP) for use in testing pesticides and chemical substances to develop data for submission to the Agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and section 408 of the Federal Food, Drug and Cosmetic (FFDCA) (21 U.S.C. 346a). Prior to April 22, 2010, OCSPP was known as the Office of Prevention, Pesticides and Toxic Substances (OPPTS). To distinguish these guidelines from guidelines issued by other organizations, the numbering convention adopted in 1994 specifically included OPPTS as part of the guideline's number. Any test guidelines developed after April 22, 2010 will use the new acronym (OCSPP) in their title.

The OCSPP harmonized test guidelines serve as a compendium of accepted scientific methodologies and protocols that are intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting the test, and is also used by EPA, the public, and the companies that are subject to data submission requirements under TSCA, FIFRA, and/or the FFDCA. As a guidance document, these guidelines are not binding on either EPA or any outside parties, and the EPA may depart from the guidelines where circumstances warrant and without prior notice. At places in this guidance, the Agency uses the word "should." In this guidance, the use of "should" with regard to an action means that the action is recommended rather than mandatory. The procedures contained in this guideline are strongly recommended for generating the data that are the subject of the guideline, but EPA recognizes that departures may be appropriate in specific situations. You may propose alternatives to the recommendations described in these guidelines, and the Agency will assess them for appropriateness on a case-by-case basis

For additional information about these test guidelines and to access these guidelines electronically, please go to http://www.epa.gov/ocspp and select "Test Methods & Guidelines" on the left side navigation menu. You may also access the guidelines in http://www.regulations.gov grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, and EPA-HQ-OPPT-2009-0576.

OCSPP 850.4400: Aquatic plant toxicity test using *Lemna* spp.

(a) **Scope**—

(1) **Applicability.** This guideline is intended to be used to help develop data to submit to EPA under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 346a).

(2) **Background.** The source materials used in developing this harmonized OCSPP test guideline include OPPT guideline under 40 CFR 797.1160 *Lemna* Acute Toxicity Test; the OPP Non-target Plants: Growth and Reproduction of Aquatic Plants - Tiers 1 and 2 Standard Evaluation Procedure; OPP 122-2 Growth and Reproduction of Aquatic Plants (Tier 1), OPP 123-2 Growth and Reproduction of Aquatic Plants (Tier 2) (Pesticide Assessment Guidelines Subdivision J—Hazard Evaluation: Nontarget Plants); OPP Pesticides Reregistration Rejection Rate Analysis: Ecological Effects; and ASTM E 1415-91, Standard guide for conducting static toxicity tests with *Lemna gibba* G3.

(b) **Purpose**. This guideline is intended for use in developing data on the toxicity of chemical substances and mixtures ("test chemicals" or "test substances") subject to environmental effects test regulations. This guideline prescribes test procedures and conditions using the freshwater vascular aquatic plants *Lemna gibba* or *Lemna minor* to develop data on the phytotoxicity of test substances. The Environmental Protection Agency will use data from these tests in assessing the hazard and risks a test substance may present in the aquatic environment. This guideline should be used in conjunction with OCSPP 850.4000 (Background and special considerations for conducting ecological effects tests with terrestrial and aquatic plants, cyanobacteria, and terrestrial soil core microcosms), which provides general information and overall guidance for the plant test guidelines and OCSPP 850.1000 (Background and special considerations for conducting ecological effects tests with aquatic and sediment-dwelling fauna and aquatic microcosms), which provides general information for conducting toxicity tests in an aqueous matrix.

(c) **Definitions.** The definitions in OCSPP 850.1000 and OCSPP 850.4000 are applicable to this guideline. In addition, the more specific definitions in this paragraph also apply:

Colony is an aggregate of mother and daughter fronds attached to each other.

Frond is a single *Lemna* "leaf-like" structure.

Frond mortality refers to dead fronds which are identified by a total discoloration (yellow, white, black, or clear) of the entire frond.

(d) **General considerations—**

(1) **Summary of the test**. Organisms of a particular species of duckweed (*Lemna gibba* or *Lemna minor*) are maintained in test vessels containing nutrient medium alone and nutrient medium to which the test substance has been added. Over an exposure period of 7 days, data on population growth are obtained on a regular basis. In addition to measurements of effects on frond number, effects on frond size (dry weight or frond area)

are determined. The test is designed to determine the quantity of test substance that results in a 50 percent inhibition (IC_{50}) in yield and average growth rate based on number of fronds and yield, and average growth rate based on frond size (dry weight or frond area), and to determine the no observed effect concentration (NOEC) for these effect measures. The results are used to establish toxicity levels, evaluate hazards or risks to aquatic vascular plants, and to indicate if further testing at a higher tier is necessary. Note historically in OCSPP pesticide and industrial chemical guidelines the term EC_x was used to cover both the current OCSPP 850.4000 definition of EC_x (concentration where *x* percent (*x*%) of the population exhibit the effect (*e.g.*, mortality)) and IC_x (concentration resulting in an *x*% decrease or inhibition effect on an attribute of the population (*e.g.*, growth rate)).

(2) **General test guidance.** The general guidance in OCSPP 850.4000 and OCSPP 850.1000 applies to this guideline except as specifically noted herein.

(3) **Range-finding test.** A range-finding test is usually conducted to establish the appropriate test solution concentrations for the definitive test. In the range-finding test, the test organisms are exposed to a series of widely-spaced concentrations of the test substance (*e.g.*, 0.1, 1.0, 10, 100 milligrams per liter (mg/L), *etc.*), usually under static conditions. The details of the range-finding test do not have to be the same as the definitive testing in that there are no replicates, and the number of test organisms used, and duration of exposure may be less than in definitive testing. In addition, the types and frequency of observations made on test organisms are not as detailed or as frequently observed as that of a definitive test and results are analyzed using nominal concentrations. However, the range-finding test will be more useful the greater the similarity between the range-finding and the definitive test.

(4) **Definitive test**. The goal of the definitive test is to determine for *Lemna* sp. the concentration-response curve for yield and growth rate, and the median inhibition concentration (IC_{50}) value for each of these responses (with 95% confidence interval and standard error). If possible the slopes of the concentration-response curves, associated standard errors, and the 95% confidence intervals of the slopes should also be determined. However, at a minimum, the full concentration-response curve (curve between IC_{05} and IC_{90}) is determined for the most sensitive measure of effect using a minimum of five concentrations of the test chemical, plus appropriate controls. For a satisfactory test, the lowest treatment concentration is below the IC_{50} value for all measures of effect. Analytical confirmation of test concentrations is performed as described in OCSPP 850.1000. A summary of test conditions is provided in Table 2 in paragraph (g) of this guideline and validity elements for an acceptable definitive test are listed in Table 3 in paragraph (h) of this guideline.

(5) **Limit test**. In some situations, it is only necessary to ascertain that the 7-day IC_{50} value for yield, area under the growth curve, and growth rate are above a certain limit concentration, and that at this limit concentration there is no observable adverse effect. For pesticides a limit test has also been referred to as a Tier I test or Maximum Challenge Concentration test. In a *Lemna* sp. limit test, at least four replicate test vessels are exposed to a single "limit concentration," with the same number of test vessels containing

the appropriate control solution(s). The multiple-concentration definitive test may be waived if the following two conditions are met for all measures of effect (yield and average specific growth rate based on number of fronds and frond size (dry weight or frond area). First, the "limit" treatment response is both statistically less than a 50% decrease from the control response (*i.e.*, IC_{50} values > limit concentration), and second, limit treatment responses are not significantly reduced (or inhibited) as compared to the control response (*i.e.*, NOEC $\geq$ limit concentration). For most industrial chemicals 1,000 mg/L or the limits of water solubility or dispersion are considered appropriate as the limit concentration. For pesticides the limit concentration is equivalent to the maximum label rate (pounds of active ingredient per acre (lbs a.i./A)) directly applied to a one acre pool that is 6 inches deep (21,280 cubic feet (ft^3) or 602,581 liters). For example, a 1 lb a.i./A (or 453,592 milligrams (mg) a.i. per acre) application rate and assuming a water density of 1 gram per milliliter would have a limit concentration of 0.75 mg a.i./L. Except for the number of treatment groups, an acceptable limit test follows the same test procedures, is the same duration, and has the same number of controls as the multi-concentration definitive test (Table 3 in paragraph (h) of this guideline). Acceptable limit tests like definitive tests include analytical confirmation of the test exposure concentration.

(e) **Test standards—**

(1) **Test substance**. For industrial chemicals, the substance to be tested should be technical grade unless the test is designed to test a specific formulation, mixture, or end-use product. For pesticides, the use of the typical end-use product (TEP) instead of the technical grade active ingredient (TGAI) is preferred for all aquatic plant phytotoxicity tests. If there is more than one TEP with the same inert substances, the one with the highest percent a.i. and/or the one most commonly used should be tested. If there is more than one TEP with different inert substances, a TEP representative of each different inert substance should be tested in the range-finding test and at a minimum the most sensitive one tested in the definitive or limit test. Adjuvants are not used with TEP or TGAI testing of algae. OCSPP 850.1000 and OCSPP 850.4000 list the type of information that should be known about the test substance before testing, and discuss methods for preparation of test substances.

(2) **Test duration.** The duration of the test is 7 days.

(3) **Test organism—**

(i) **Species.** The test species is *L. gibba* G3 or *L. minor*. *L. gibba* has been widely used for testing with pesticides and other chemicals in the United States, while *L. minor* has been used more frequently for testing of environmental samples and in Europe. The identity of the organism should be verified using an appropriate taxonomic key and it is also desirable to identify the clone (see paragraph (j)(1) of this guideline).

(ii) **Source.** Axenic cultures may be obtained from laboratory cultures or commercial sources.

(iii) **Age and condition.** The plants used in testing should be from stock cultures that have been actively growing in growth medium under the same conditions as used in the test for at least eight weeks immediately preceding the start of the test. Plants should be aseptically transferred on a regular schedule (such as weekly) to fresh growth medium. Long-term maintenance of cultures on a solid medium containing 1% agar in sterile Petrie dishes or test tubes may be desirable. However for a satisfactory test, the plants used to initiate toxicity tests are from a liquid culture. Plants used in a test should be randomly selected from cultures which are between 7 and 12 days old.

(iv) **Culturing procedures.** *Lemna* spp. cultures should be maintained in growth medium using the culturing procedures described in the reference in paragraph (j)(1) of this guideline. The cultures should be maintained under the same conditions as used for testing.

(4) **Administration of test substance—**

(i) **Preparation of test solutions—**

(A) **Stock solutions or direct addition.** Test solutions are prepared by adding the test substance directly to the nutrient medium or by addition of a stock solution to the nutrient medium. Typically, a stock solution of the test substance is prepared and aliquots of the stock solution added to nutrient medium to obtain the desired test concentrations. Guidance for preparation of test solutions, especially for difficult or low solubility test substances, is provided in OCSPP 850.1000.

(B) **Solvents.** The recommended solvent for *Lemna* spp. toxicity tests is N,N-dimethyl-formamide, as solvents such as acetone can cause stimulation of bacterial growth. The concentration of solvent should preferably be the same in all test treatments and should not exceed 0.1 milliliter per liter (mL/L).

(C) **Exposure technique.**

(1) For pesticides, this test should be conducted using the static renewal exposure technique. For industrial chemicals, this test may be conducted using a static, static renewal, or flow-through with guidance on the selection of the appropriate exposure technique based on the stability of the test substance as provided in OCSPP 850.1000. To conduct tests with duckweed using the flow-through exposure technique see the references in paragraphs (j)(2), (j)(4) and (j)(13) of this guideline, which may be necessary when testing volatile industrial test substances.

(2) Static renewal of test solutions. For static renewal tests, the plants should be transferred to fresh test solutions of initial test concentration levels at intervals necessary to maintain the test

concentrations (for example, on days 3 and 5 or on days 2 and 4). Transfer should be done using aseptic technique in a clean, draft-free area as quickly as possible to minimize contamination of the colonies.

(3) When a substance is know to have a tendency to vaporize, closed test flasks with increased head-space may be used. Attempts should be made to determine the amount of the substance which remains dissolved in solution, and extreme caution is advised when interpreting results of test with volatile chemicals using closed systems.

(ii) **Treatment levels.** At a minimum five test solution concentrations are tested for multi-concentration definitive testing, plus the appropriate control(s). A range-finding test can be used to establish the appropriate test solution concentrations for the definitive test (see paragraph (d)(3) of this guideline). For scientifically sound estimates of a given point estimate (*e.g.*, IC_{50}), test substance concentrations should immediately bracket the point estimate(s) of concern. The concentrations should be a twofold geometric progression (*e.g.*, 0.1, 0.2, 0.4, 0.8, and 1.6 milligrams per liter (mg/L)). While a twofold progression is preferred, threefold and fourfold progressions are acceptable. If a fourfold or higher series progression is used, the rationale for using this large an interval between concentrations and the effect on the accuracy and reproducibility of the point estimate and NOEC should be provided. For an acceptable study, the lowest test treatment level should be lower than the IC_{50} values for yield and average specific growth rate based on number of fronds and frond size. The lack of a NOEC for an effect measure is not critical as long as the response-curve for the effect measure is acceptable for calculation of the 5% inhibition concentration (IC_{05}). It is recommended that one or two additional test concentrations in the lower tail of the concentration-response curve of the most sensitive endpoint be added to insure bracketing of both the most sensitive IC_{50} value and the most sensitive NOEC (or IC_{05}) value. For a limit test, there is single treatment concentration, plus the appropriate control(s). Guidance on the limit concentration is provided in paragraph (d)(5) of this guideline.

(iii) **Introduction of test organisms.**

(A) In preparation for the test, containers are filled with appropriate volumes of nutrient medium and/or the test solutions. The test is initiated by introducing *Lemna* sp. fronds into each of the test vessels within 30 minutes of addition of test substance to test solutions. Plants should be placed in the test vessels using a sterile inoculating loop or hook, Nitex screen, or other aseptic technique. The plants should be impartially or randomly distributed among the test vessels in such a manner that test results show no significant bias from the distributions. The test vessels are then immediately placed in a growth chamber or the controlled laboratory environment.

(B) Test vessels within the testing area are positioned in a random manner or in a way in which appropriate statistical analyses can be used to determine the variation due to placement.

(5) **Controls.**

(i) Every test includes a negative control treatment consisting of the same nutrient medium, number of test organisms, environmental conditions, and procedures as the treated test vessels except that none of the test substance is added. In addition, vehicle (solvent) controls are also included if a solvent is used to dissolve or suspend the test substance.

(ii) At test termination the control coefficient of variation for yield should generally be less than 20% and substantially less than 20% for growth rate, which is a logarithmically-transformed variable.

(iii) For a satisfactory test the doubling time of number of fronds in the control should be less than 2.5 days (60 hours), corresponding to approximately a seven-fold increase in seven days and an average specific growth rate of 0.275 per day (d^{-1}).

(6) **Number of test organisms and replicates.**

(i) The minimum number of replicates per treatment and control(s) is four, each containing three to five plants (consisting of three to four fronds per plant). Plants of similar size and appearance should be selected, and the number of plants and number of fronds should be identical in each test vessel. A total of at least 12, but no more than16 fronds, per test vessel are recommended (*e.g.* three 4-frond plants and one 3-frond plant could be used, for a total of 15 fronds).

(ii) For determination of initial dry weight of fronds at test initiation, an additional control set of replicates are created at test initiation using plants of similar size and appearance as those selected for the test. The number of replicates should be equivalent in number to that used for the test.

(7) **Facilities, apparatus and supplies—**

(i) **Containers for culturing and testing.** Glass beakers (250 - 1000 milliliters (mL)), 250 mL flat-bottomed test tubes, and Erlenmeyer flasks (250 - 500 mL) have been used successfully. For a satisfactory test, test vessels should be large enough to hold the *Lemna* colonies without crowding (*i.e.*, space and nutrient availability should not be growth limiting factors) for the duration of the test. Test vessels should be covered to keep out extraneous contaminants and to reduce evaporation of test solutions. Containers and covers that may contact the test solution should be chosen to minimize sorption of test substances, and not contain substances that can be leached or dissolved into aqueous solutions in quantities that can affect the test results. Beakers may be covered with a clear watch glass while Erlenmeyer flasks may be covered with foam plugs, stainless steel caps,

Shimadzu enclosures, glass caps or screw caps. (The acceptability of foam plugs should be investigated prior to use because some brands have been found to be toxic). For a satisfactory test, all test vessels and covers in a test are identical.

(ii) **Growth chamber or laboratory environment.**

(A) A controlled environment growth chamber or an enclosed laboratory area capable of maintaining the specified number of test vessels and able to maintain the air temperature, light intensity rate, and photoperiod specified in this guideline.

(B) Facilities should be well ventilated and free of fumes that may affect the test organisms. Construction materials and equipment that may contact the stock solution, test solution, or nutrient medium should be chosen to minimize sorption of test substances and not contain substances that can be leached or dissolved into aqueous solutions in quantities that can affect the test results. Refer to OCSPP 850.1000 for additional information on appropriate construction materials.

(iii) **Environmental monitoring equipment.** Equipment for determination of test environmental conditions (*e.g.*, pH meter, photosynthetically active radiation (PAR) light sensor, *etc*.)

(iv) **Cleaning and sterilization.** Apparatus for sterilizing glassware, preparing sterile nutrient media, and maintaining aseptic technique during culturing and testing. All glassware and equipment used in *Lemna* spp. culturing or testing is to be cleaned and sterilized prior to use. The Nitex screen or inoculating loops used for transferring the *Lemna* sp. should be discarded after use or thoroughly cleaned and sterilized before reuse.

(v) **Nutrient media and diluent.** Different media are recommended for culturing and testing *L. gibba* and *L. minor*.

(A) **Medium for *L. gibba*.** 20X-AAP medium is recommended for maintaining *L. gibba* cultures and for use as the diluent in the preparation of the various test solution concentrations. Medium is sterilized by autoclaving or filtering (0.22 micrometer (μm) filter). Preparation of this medium is described under paragraph (j)(1) of this guideline and in Table 1. Water used for preparation of nutrient medium should be of reagent quality (*e.g.*, ASTM Type I water). If prepared in advance, medium should be stored under refrigeration in the dark at 4 degrees Celsius (^{o}C).

Table 1.—Preparation of 20X-AAP Medium

Add 20 mL of each of the macronutrient stock solutions and 20 mL of the micronutrient stock solution listed in this table to approximately 800 mL reagent grade water and then dilute to 1 liter (L).
Macronutrient stock solutions are made by dissolving the following chemicals into 500 mL of reagent grade water: Sodium nitrate ($NaNO_3$) - 12.750 grams (g) Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) - 6.082 g Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) - 2.205 g Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) - 7.350 g Potassium phosphate (K_2HPO_4) - 0.522 g Sodium bicarbonate ($NaHCO_3$) - 7.500 g
The micronutrient stock solution is made by dissolving the following chemicals into 500 mL of reagent grade water: Boric acid (H_3BO_3) - 92.760 milligrams (mg) Manganese chloride tetrathydrate ($MnCl_2 \cdot 4H_2O$) - 207.690 mg Zinc chloride ($ZnCl_2$) - 1.635 mg Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) - 79.880 mg Cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$) - 0.714 mg Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$) - 3.630 mg Copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$) - 0.006 mg [Typically must be prepared by serial dilution]. Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA \cdot 2H_2O$) - 150.000 mg
Adjust pH to 7.5 ± 0.1 with 0.1 Normal (*N*) or 1.0 *N* sodium hydroxide (NaOH) or hydrochloric acid (HCl). Filter the media into a sterile container through a 0.22 µm membrane filter. Store medium in the dark at approximately 4 degrees Celsius (ºC) until use.

(B) **Medium for *L. minor*.** A modification of the Swedish standard (Standardiseringen I Sverige (SIS)) *Lemna* spp. medium is recommended for culturing and testing with *L. minor*. Preparation of this medium is described in references in paragraphs (j)(5) and (j)(8) of this guideline.

(vi) **Equipment to observe plants.** A lighted magnifying lens, dissecting microscope, or other device may be used to facilitate observations of the fronds.

(8) **Environmental conditions.** Environmental conditions during the test should be maintained as follows:

(i) **Temperature.** Test solution temperature should be 25 ± 2 ^{o}C throughout the duration of the test.

(ii) **Lighting and photoperiod.** Continuous lighting should be used to provide a light intensity in the range of 57 - 90 micromoles per meter square per second (µmol/m^2/s). For cool-white fluorescent lighting, this is approximately equivalent to 4200 to 6700 lux. Warm-white or cool-white fluorescent lighting has been used for testing with *Lemna* spp. Additional information on the use of lighting in plant toxicity tests can be found in the references given in OCSPP 850.4000.

(iii) **Nutrient medium pH.** Prior to use, the pH of the nutrient medium should be adjusted to 7.5 ± 0.1. Adjustment of pH can be accomplished by adding acid or base prior to the addition of the test substance. The pH may be adjusted in stock solutions to match the pH of the nutrient medium if pH change does not affect the stability of the test substance in the stock solution or test solution. Hydrochloric

acid (HCl) and sodium hydroxide (NaOH) may be used for this adjustment if warranted. The pH should not be adjusted after addition of the test substance or stock solution into the test medium. Test solution pH may vary from the nutrient medium after the addition of the test substance and/or vehicle (if used). Any such changes should be recorded but not adjusted. Chelating agents, such as ethylenediamine-tetraacetic acid, are present in the 20X-AAP medium to ensure that trace nutrients will be available to the *L. gibba* fronds.

(9) **Observations—**

(i**) Measurement of test substance.** Analytical confirmation of dissolved test concentrations is performed at test initiation and at test termination and as described for renewal tests in OCSPP 850.1000. Samples for analysis should be collected as described in OCSPP 850.1000, with the following exception: if there is insufficient volume at test termination to perform the analysis, the contents of the replicate test vessels may be pooled after the plants have been removed for observations. The analytical methods used to measure the amount of dissolved test substance in a sample are validated before beginning the test, as described in OCSPP 850.1000.

(ii) **Test solution appearance.** Observations are made daily on test substance solubility (*e.g.*, surface slicks, clarity, precipitates, or material adhering to the sides of the test vessels) and recorded.

(iii) **Dilution water quality.** The dilution water source used to prepare media should be periodically analyzed to document and characterize the hardness, alkalinity, pH, conductivity, total organic carbon (TOC) or chemical oxygen demand magnitude and variability, and to ensure that pesticides, PCBs and toxic metals are not present at concentrations that are considered toxic. See OCSPP 850.1000 for guidance on dilution water.

(iv) **Environmental conditions—**

(A) **Temperature.** It is impractical to measure the temperature of the solutions in the test vessels while maintaining axenic conditions. Therefore, one or two extra test vessels may be prepared for the purpose of measuring the solution temperature during the test. Alternatively, hourly measurements of the air temperature (or daily measurements of the maximum and minimum) are acceptable. Because the vessels are placed in an environmental chamber or incubator, the air temperature is more likely to fluctuate than the water temperature.

(B) **Light intensity.** Light intensity should be monitored at test initiation at the approximate level of the test solution at each test vessel position in the growth chamber or laboratory. If it is suspected that light intensity has changed by 15% or more, monitoring of light intensity should be conducted daily. A photosynthetically active radiation (PAR) sensor

should be used to measure light quality. The light intensity at each position should not differ by more than 15% from the selected light intensity. Because illumination may vary at different positions, and since this environmental parameter has an important influence on duckweed growth, it is recommended that the test vessels be randomly re-positioned on a regular schedule (*e.g.*, daily) to minimize spatial differences.

(C) **pH.** The pH in the control(s) and test solutions should be measured. This measurement is made on the bulk test solutions at the beginning of each renewal period and at the end of each renewal period on samples of pooled replicates of each test treatment provided none of the replicates appear to be "outliers" with respect to growth. In the case of outlier growth response individual pH measurements should be made in each test vessel. For a flow-through test for industrial chemicals see OCSPP 850.1000 for guidance on frequency and sampling scheme for pH.

(v) **Measures of effect—**

(A) **Frond number.** Using a dissecting microscope or lighted magnifying lens the number of fronds in each test vessel is determined and recorded at least every three days during the test and at test termination (*e.g.* days 3, 5, and 7 or days 2, 4, and 7). A frond is counted regardless of size as long as it is visible adjacent to the parent frond. Any bud which is visible when viewed under a hand lens or dissecting microscope should be counted as a frond.

(B) **Frond size.** Acceptable indicators of frond size include determination of dry weight and/or measurement of frond area. Frond size determined using dry weight, is made at test initiation and test termination. Frond size determined using frond area is determined at a minimum at test initiation and termination.

(1) **Dry weight.** Dry weight is a destructive procedure. In order to evaluate the increase in dry weight over the course of the test, a representative sample of fronds at test initiation should be processed (see paragraph (e)(6)(ii) of this guideline), using the same drying method as used at test termination, to establish the treatment and control mean dry weight of fronds at test initiation. To determine dry weight, the plants (including roots and root fragments) for a test vessel (replicate) are removed from the test solution, rinsed with distilled or deionized water, blotted to remove excess water, and placed in previously dried, tared weighing pan for that replicate. The plants should be dried at approximately 60 ^{o}C, cooled in a dessicator, and weighed to the nearest 0.1 milligram (mg). The drying process is repeated until constant weight is obtained.

(2) **Frond area.** Frond area for a test vessel (replicate) is determined at a minimum at test initiation and test termination but may also be determined at various intervals during the test. Frond area can be determined using image analysis, in which an image of each plant in a test vessel (replicate) is captured using a video camera and then digitized. An alternative procedure is to photograph the test vessel (replicate) from the top, cut out the silhouette of the plants, and determine the area using a leaf area analyzer or graph paper.

(C) **Appearance or condition.** The appearance or condition (*e.g.*, decrease in size, necrosis, chlorosis, sinking of fronds, or other abnormalities) of plants in each test vessel (replicate) is determined at least every three days during the test and at test termination (*e.g.* days 3, 5, and 7 or days 2, 4, and 7).

(D) **Phytostatic and phytocidal effects.** At test termination, it may be desirable to determine phytostatic and phytocidal effects, *e.g.* whether or not plants that were inhibited during the exposure period are able to resume growth when transferred to test substance-free medium. This optional procedure may be conducted as described in the reference in paragraph (j)(6) of this guideline.

(f) **Treatment of results—**

(1) **Determination of crowding.** For each treatment and control vessel (replicate) calculate the dry weight per frond (w_{frond}) at test termination using Equation 1. If nutrient availability or space was limiting to growth, the dry weight per frond may be observed to increase as the number of fronds decreases.

$$w_{frond} = w_{total} / n \qquad \textbf{Equation 1}$$

where:

w_{total} = total dry weight biomass in a test vessel at test termination; and

n = the number of fronds in the test vessel at test termination.

(2) **Response variable calculations.** There are two response variables calculated from number of fronds and frond size: yield and average specific growth rate.

(i) **Yield calculations.** For each treatment and control test vessel (replicate) calculate the yield by subtracting the initial number of fronds, frond area, or dry weight from the number of fronds, frond area, or dry weight, respectively, obtained at test termination (Equation 2).

$$Y = b_1 - b_0 \qquad \textbf{Equation 2}$$

where:

Y = yield of observed biomass (number of fronds or dry weight or frond area)

b_0 = biomass (number of fronds or dry weight or frond area, respectively) at test initiation

b_1 = biomass (number of fronds or dry weight or frond area, respectively) at test termination

(ii) **Average specific growth rate calculations.** Average specific growth rate (sometimes called relative growth rate) is the rate of growth over a given time interval. The growth rate for number of fronds, frond area, and dry weight for each test vessel (replicate) over a given time interval is calculated as given in Equation 3. At a minimum, the average specific growth rate is calculated for the time interval between test initiation and termination.

$$\bar{r}_{i-j} = \frac{\ln(b_j) - \ln(b_i)}{t} \qquad \textbf{Equation 3}$$

where:

$\bar{r}_{i-j}$ = average specific growth rate per day (day^{-1}) of observed biomass (number of fronds or dry weight or frond area) from time i to j.

b_i = observed biomass (number of fronds or dry weight or frond area, respectively) at beginning of the observation interval, time i

b_j = observed biomass (number of fronds or frond area or dry weight, respectively) at end of the observation interval, time j

t = time period from i to j in days

(3) **Summary statistics—**

(i) **Environmental conditions.** Calculate descriptive statistics (mean, standard deviation, coefficient of variation, minimum, maximum) by treatment level for temperature and pH. Calculate descriptive statistics (mean, standard deviation, coefficient of variation, minimum, maximum) by test vessel position for light intensity.

(ii) **Test substance concentration.** For each treatment level compare the test substance concentration at the start and end of each renewal period. For a stable test substance calculate the time-weighted average concentration. For industrial

chemicals if the test substance was unstable calculate a rate of decline and the associated time-weighted mean concentration (*e.g.*, exponential decay calculate the area under the exponential decay concentration curve divided by the total exposure days). Calculate descriptive statistics such as the time-weighted mean, standard deviation, minimum, maximum, and coefficient of variation for each test vessel and treatment level. For pesticides under unstable test substance conditions, use the mean test substance concentration in solution measured at test initiation and in bulk renewal solutions for calculating concentration-response and NOEC-LOEC values. Such an approach is used rather than the exponential time-weighted averaged because of the exposure estimate currently used by OPP for calculating risk estimates.

(iii) **Number of fronds.** For each treatment level and observation time calculate the mean, standard deviation, and coefficient of variation for number of fronds. Calculate the mean treatment yield and average specific growth rate based on changes in number of fronds from test initiation to test termination.

(iv) **Frond size—**

(A) **Dry weight.** Calculate the mean treatment yield and average specific growth rate based on changes in dry weight from test initiation to test termination.

(B) **Frond area.** For each treatment level and observation time calculate the mean, standard deviation, and coefficient of variation for frond area. Calculate the mean treatment yield and average specific growth rate based on changes in frond area from test initiation to test termination.

(v) **Appearance and condition.** Morphological symptoms of plant injury should be summarized in tabular form by time of observation, treatment, and replicate. Definition of any index values used for morphological symptoms, indicating the severity of the symptom(s), should be provided.

(4) **Percent inhibition.** For yield and average specific growth rate for each response variable calculate the percent inhibition (%I) at each treatment level using Equation 4.

$$\%I = \frac{(C - X)(100)}{C} \qquad \textbf{Equation 4}$$

where:

C = the control mean response value (yield or average specific growth rate); and

X = the treatment mean response value (yield or average specific growth rate, respectively). Stimulation is reported as negative %I.

(5) **Doubling time of control.** The doubling time (T_d) of the number of fronds in the control is calculated using Equation 5.

$$T_d = \ln(2) \Big/ \bar{r}_{Control} \qquad \textbf{Equation 5}$$

where:

$\bar{r}_{Control}$ = mean of the control average specific growth rate test vessel values from paragraph (f)(1)(ii) of this guideline.

(6) **Limit test—**

(i) **IC_{50} values.** To ascertain that the yield and average specific growth rate IC_{50} values, based on number of fronds and frond size, occur above the "limit" concentration, a one-sided test which compares the difference between two sample groups to a fixed value (or difference) is performed for each of these response measures. For a comparison of sample means, the difference defining the IC_{50} compared to controls is operationally defined as a 50% reduction or inhibition from the control sample mean (Equation 6). The null hypothesis (H_0) stated in terms of true population parameters is that the difference of the "limit" treatment response (μ_{limit}) from the control mean response ($\mu_{control}$) is greater than or equal to a 50% inhibition or reduction, compared to the control (*i.e.*, H_0: $\mu_{control} - \mu_{limit} \geq \delta_0$). The alternative hypothesis (H_A) is that this difference is less than a 50% reduction, compared to the control (H_A: $\mu_{control} - \mu_{limit} < \delta_0$). An example of a parametric two-sample comparison test of this is the Student's t-test. If the null hypothesis is rejected, the inhibition level for the given response measure (*i.e.* yield and average specific growth rate based on frond number and size) in the limit treatment as compared to the control is declared to be less than 50% (*i.e.*, IC_{50} > limit concentration). If the null hypothesis is not rejected, the limit treatment as compared to the control response is declared to be 50% or greater (*i.e.*, $IC_{50} \leq$ limit concentration).

$$\delta_0 = \left(\bar{x}_{control}\right) \times \left(p/100\right) \qquad \textbf{Equation 6}$$

where:

δ_0 = difference between two parameters, defined in this case as a *p*% reduction from the control sample mean;

$\bar{x}_{control}$ = control sample mean response (*e.g.*, yield and average mean biomass); and

p = percent reduction from the control sample mean, which is 50 in the case of the IC_{50}.

(ii) **NOEC.** To ascertain that there is no observable effect at the limit treatment (*i.e.*, NOEC $\geq$ limit dose) for a given response measure (yield and average specific growth rate based on frond number and size), the limit treatment response is compared to the control treatment response using a one-sided two-sample parametric or nonparametric test, as appropriate (see OCSPP 850.4000). The minimum significant difference detectable by the test or a similar estimate of the sensitivity of the test should be determined and reported.

(iii) M**ultiple-concentration definitive testing.**

(A) A multiple-concentration definitive test is performed if either the effect or inhibition level for one or more response measures (*i.e.*, yield and average specific growth rate based on frond number and size) in the limit treatment as compared to the control response at test termination are declared to be 50% or greater effect (*i.e.*, the null hypothesis is not rejected) or the NOEC is less than the limit concentration.

(B) Multiple-concentration definitive testing may be waived if at test termination the "limit" treatment response is both statistically less than a 50% decrease from the control response and there is no observable adverse effect from the control response for all measures of effect (yield and average specific growth rate based on frond number and size).

(7) **Multiple-dose definitive test—**

(i) **Concentration-response curve, slope and IC_{50}.** For dose-response tests statistical procedures are employed to calculate the IC_{50} value (standard error and 95% confidence interval) for yield and growth rate based on number of fronds and frond size (dry weight or frond area) at test initiation and test termination. If a concentration-response curve model was fit to the data to determine an IC_{50} value, the model parameters (*e.g.*, slope) and their uncertainty estimates (*e.g.*, standard error) should be recorded. The response values for each test vessel, not the mean response for each treatment level, should be used in fitting the model. Where the concentration-response range tested does not result in the determination of a definitive IC_{50} value for a given response measure, test and document that the IC_{50} value is above the highest treatment level tested (see the statistical guidance in paragraph (f)(6) of this guideline). Such an event may arise if one of the other response measures is much more sensitive, and while the full response curve for that response measure is captured too many additional treatments would be needed to capture the full response relationship for the other less sensitive response measure(s).

(ii) **NOEC.** The 7-day NOEC for yield and growth rate based on number of fronds and frond size (dry weight or frond area) are determined (see OCSPP 850.4000). If a 7-day NOEC value can not be determined for a given response measure, the concentration at which there is a 5% inhibition (*i.e.*, an IC_{05} value for yield or growth rate) is estimated and used in place of the given NOEC. The

standard error and 95% confidence interval should also be calculated for the IC_{05} value. For industrial chemicals, the specific IC_x used in place of a NOEC that can not be determined will vary, consult with the Agency. Methods, assumptions, and results of the statistical approaches used should be recorded.

(iii) **Statistical methods.** Statistical procedures for modeling continuous toxicity data are available and should be used (see references in paragraphs (j)(3), (j)(7) and (j)(12) of this guideline). Additional discussion about endpoints and statistical procedures is found in OCSPP 850.1000 and OCSPP 850.4000.

(g) **Tabular summary of test conditions.** Table 2 lists the important conditions that should prevail during the definitive test. Except for the number of treatment levels, Table 2 also lists the important conditions that should prevail during a limit test. Meeting these test conditions will greatly increase the likelihood that the completed test will be acceptable or valid.

Table 2.—Summary of Test Conditions for the *Lemna* sp. Toxicity Test

Test type	Static renewal (pesticides) Static, static renewal, or flow through (industrial chemicals)
Test duration	7 days
Test matrix	Synthetic growth medium
Temperature	25 ± 2 °C
Light quality	Warm-white or cool-white fluorescent
Light intensity	57 - 90 µmol/m^2/s
Photoperiod	Continuous
Test vessel size	Sufficient to prevent crowding (*e.g.*, 250 - 1000 mL beakers or flasks)
Age of inoculum	From healthy stock cultures 7 -12 days old
Size of inoculum	12 -16 fronds total, with the same number of plants and fronds in each test vessel
Number of replicate test vessels per concentration	Four (minimum)
Test concentrations	Unless performing limit test, minimum of 5 test concentrations plus appropriate controls
Test concentration preparation	Aqueous solutions prepared by adding test substance to synthetic nutrient medium, directly or via vehicle
Measures of effect (measurement endpoints)	IC_{50} and NOEC (or IC_{05}) values for yield, and average specific growth rate based on frond number IC_{50} and NOEC (or IC_{05}) values for yield and average specific growth rate based on frond size (dry weight or frond area)

(h) **Test validity elements.** This test would be considered to be unacceptable or invalid if one or more of the conditions in Table 3 occurred or one or more performance objectives in Table 3 were not met. This list should not be misconstrued as limiting the reason(s) that a test could be found unacceptable or invalid. However, except for the conditions listed in Table 3 and in OCSPP 850.4000 and OCSPP 850.1000, it is unlikely a study will be rejected when there are

slight variations from guideline environmental conditions and study design unless the control organisms are significantly affected, the precision of the test is reduced, the power of a test to detect differences is reduced, and/or significant biases are introduced in defining the magnitude of effect on measurement endpoints as compared to guideline conditions. Before departing significantly from this guideline, the investigator should contact the Agency to discuss the reason for the departure and the effect the change(s) will have on test acceptability. In the test report, all departures from the guideline should be identified, reasons for these changes given, and any resulting effects on test endpoints noted and discussed.

Table 3.—Test Validity Elements for the *Lemna* Toxicity Test

1. All test vessels and closures were not identical.
2. The duckweed plants were not impartially or randomly assigned to the test vessels.
3. A medium (untreated) control [and solvent (vehicle) control, when a solvent was used] was not included in the test.
4. For testing with industrial chemicals, a surfactant or dispersant was used in the preparation of a stock or test solution.
5. The concentration of solvent in the range used affected growth of the test species.
6. A minimum of five test concentrations were not used in the definitive test.
7. Controls were contaminated with the test substance.
8. Temperature and light intensity were not measured as specified during the test.
9. The doubling time of number of fronds in the control exceed 2.5 days.
10. The lowest test concentration level was not less than the 7-day yield and average specific growth rate IC_{50} values based on number of fronds and frond area (dry weight or frond area).

(i) **Reporting—**

(1) **Background information.** Background information to be supplied in the report consists at a minimum of those background information items listed in paragraph (j)(1) of OCSPP 850.4000.

(2) **Guideline deviations.** Provide a statement of the guideline or protocol followed. Include a description of any deviations from the test guideline or any occurrences which may have influenced the results of the test, the reasons for these changes, and any resulting effects on test endpoints noted and discussed.

(3) **Test substance.**

(i) Identification of the test substance: common name, IUPAC and CAS names, CAS number, structural formula, source, lot or batch number, chemical state or form of the test substance, and its purity (*i.e.* for pesticides, the identity and concentration of active ingredient(s)).

(ii) Storage conditions of the test chemical or test substance and stability of the test chemical or test substance under storage conditions if stored prior to use.

(iii) Methods of preparation of the test substance and the treatment concentrations used in the range-finding and definitive test, or limit test.

(iv) If a vehicle (solvent) is used to prepare stock or test substance provide: the name and source of the vehicle, the nominal concentration(s) of the test substance in the vehicle in stock solutions or mixtures, and the vehicle concentration(s) used in the treatments and solvent control.

(4) **Plant test species.**

(i) Scientific and common name, plant family, and strain.

(ii) Source and method of species and strain verification.

(iii) Culture practices, including culturing media used, and conditions.

(iv) Acclimation period, if applicable.

(v) Age (stage) of inoculum at test initiation.

(5) **Test system and conditions.** Description of the test system and conditions used in the definitive or limit test, and any preliminary range-finding tests.

(i) Description of the incubator, growth chamber, or laboratory location, type of lights and aeration or agitation of test vessels.

(ii) Description of the test container used: size, type, material, fill volume.

(iii) Number of fronds per plant and number of plants and fronds added to each test vessel at test initiation.

(iv) Number of test vessels (replicates) per treatment level and control(s).

(v) Description of the preparation of the synthetic growth media used including the preparation date, concentration of all constituents, the initial pH, and storage conditions and duration prior to use in test.

(vi) Description of the dilution water and any water pretreatment: source/type; pH; total organic carbon content; particulate matter content; metals, pesticides, and chlorine concentration. Describe the frequency and sample date(s) for documenting dilution water quality.

(vii) Methods used for treatment randomization and assignment of plants to test vessels.

(viii) Date of introduction of test organisms to test solutions and test duration.

(ix) Exposure technique and frequency of renewal if static renewal and flow rate if flow-through.

(x) The photoperiod and light source.

(xi) Methods and frequency of environmental monitoring performed during the definitive or limit study for temperature, light intensity, pH.

(xii) Methods and frequency of measuring test substance to confirm exposure concentrations.

(xiii) Methods and frequency of measuring number of fronds, frond size (dry weight or frond area), and any other symptoms.

(xiv) For the definitive and limit test, all analytical procedures should be described. The accuracy of the method, method detection limit, and limit of quantification should be given.

(6) **Results.**

(i) Tabulation of test substance analytical results by test vessel and treatment (provide raw data) and descriptive statistics (time-weighted mean, standard deviation, minimum, maximum, coefficient of variation).

(ii) Environmental monitoring data results (test solution temperature, light intensity, and pH) in tabular form (provide raw data for measurements not made on a continuous basis), and descriptive statistics (mean, standard deviation, minimum, maximum).

(iii) For preliminary range-finding tests, if conducted, the number of fronds and frond size (dry weight or frond area) at each treatment level and in the control(s). A description and count of visual phytotoxic effects, if recorded, at each treatment level and in the control(s).

(iv) For a limit test, tabulate for the limit concentration and the control(s) by replicate, the number of fronds and frond size (dry weight in representative samples or frond area) at test initiation and termination, the number of fronds and, if measured, frond area in each test vessel at each observation time during the test (provide the raw data).

(v) For the definitive test, tabulation by test vessel and treatment of the number of fronds and frond size (dry weight or frond area) at test initiation and termination, and the number of fronds and, if measured, frond area in each test vessel at each observation time during the test (provide the raw data).

(vi) For the limit and definitive tests, tabulation by test vessel and treatment of yield and average specific growth rate for number of fronds and frond area (dry weight or frond area).

(vii) For the limit and definitive tests, tabulation of the mean treatment yield and average specific growth rate values, treatment standard deviations for these variables, and the treatment %I (or stimulation) in yield and average specific growth rate relative to the control values.

(viii) For the limit and definitive test, tabulation of observed morphologic signs of toxicity (chlorosis, necrosis, mortality, pigmentation, abnormal shape).

(ix) Graphs of the concentration-response data for yield and average specific growth rate based on number of fronds and frond size (dry weight or frond area).

(x) For a limit test, provide the results of hypothesis tests.

(xi) For the limit test, provide a description of the statistical methods used including software package, and the basis for the choice of method.

(xii) For the definitive study and for those effect measures (yield and average specific growth rate for number of fronds and frond size (dry weight or frond area)) with data sufficient to fit a concentration-response relationship, tabulation of the slope of the concentration-response curve and its standard error and 95% confidence limits and any goodness of fit results.

(xiii) For the definitive test, tabulation of IC_{50} values for yield and average specific growth rate for number of fronds and frond size (dry weight or frond area).

(xiv) For the definitive test, a tabulation of the NOAEC and LOAEC for each response variable (yield and average specific growth rate based on number of fronds and frond size (dry weight or frond area)). The IC_{05} and 95% confidence interval should be reported for response data where an NOAEC could not be determined.

(xv) Description of statistical method(s) used for point estimates, including software package, for determining IC_{50} values, fitting the dose-response model, and the basis for the choice of method. Provide results of any goodness-of-fit tests.

(xvi) Description of statistical method(s) used for NOAEC and LOAEC determination, including software package, and the basis for the choice of method. If an IC_{05} value is used in place of a NOAEC provide a description of statistical method(s) used for point estimates, including software package, for determining IC_{05} values, fitting the dose-response model, and the basis for the choice of method. Provide results of any goodness-of-fit tests.

(xvii) If determined, report the phytostatic and phytocidal concentrations.

(j) **References**. The following references should be consulted for additional background material on this test guideline.

(1) American Society for Testing and Materials. ASTM E 1415-91(2004). Standard guide for conducting static toxicity tests with *Lemna gibba* G3. In Annual Book of ASTM_Standards, Vol. 11.06, West Conshohocken, PA. Current edition reapproved 2004.

(2) Bishop, W.E., and R.L. Perry, 1981. The development and evaluation of a flow-through growth inhibition test with duckweed (*Lemna minor*), In Aquatic Toxicology and Hazard Assessment, ASTM STP 737, ASTM, Philadelphia, PA, pp. 421 - 435.

(3) Bruce, R.D. and D.J. Versteeg, 1992. A statistical procedure for modeling continuous toxicity data. Environmental Toxicology and Chemistry 11:1485-1494.

(4) Davis, J.A., 1981. Comparison of static-replacement and flow-through bioassays using duckweed, *Lemna gibba* G3. EPA-560/6-81-003, U.S. Environmental Protection Agency, Washington, DC.

(5) Environment Canada, 1999. Biological Test Method: Test for Measuring the Inhibition of Growth using the Freshwater Macrophyte, *Lemna minor*. Report EPA 1/RM/37, Method Development and Application Section, Environmental Technology Centre, Environment Canada, Ottawa, Ontario.

(6) Hughes, J.S., Alexander, M.M., and Balu, K, 1988. An evaluation of appropriate expressions of toxicity in aquatic plant bioassays as demonstrated by the effects of atrazine on algae and duckweed, Aquatic Toxicology and Hazard Assessment: 10th Volume, ASTM STP 971, W.J. Adams, G.A. Chapman and W.A. Landis, eds., ASTM, Philadelphia, PA, pp. 531-547.

(7) Nyholm, N., P.S. Sorenson, K.O. Kusk, and E.R. Christensen, 1992. Statistical treatment of data from microbial toxicity tests, Environmental Toxicology and Chemistry 11:157-167.

(8) Organization for Economic Co-operation and Development, 2006. OECD Guidelines for Testing of Chemicals, Test No. 221, *Lemna* sp. Growth Inhibition Test, Adopted 23 March, 2006.

(9) U.S. Environmental Protection Agency, 1994. Pesticides Reregistration Rejection Rate Analysis: Ecological Effects, Office of Prevention, Pesticides and Toxic Substances, Washington, D.C. EPA 738-R-94-035

(10) U.S. Environmental Protection Agency, 1986. Hazard Evaluation Division Standard Evaluation Procedure, Non-target Plants: Growth and Reproduction of Aquatic Plants Tiers 1 and 2. Office of Pesticides Programs, Washington, D.C. EPA 540/9-86-134.

(11) U.S. Environmental Protection Agency, 1982. Pesticide Assessment Guidelines, Subdivision J Hazard Evaluation: Non-target plants. Office of Pesticides and Toxic Substances, Washington, D.C. EPA 540/9-82-020, October 1982.

(12) VanEwijk, P.H. and J.A. Hoekstra, 1993. Calculation of the EC50 and its confidence interval when subtoxic stimulus is present, Ecotoxicology and Environmental Safety 25:25-32.

(13) Walbridge, C.T., 1977. A flow-through testing procedure with duckweed (*Lemna minor* L.), EPA -600/3-77-108, U.S. Environmental Protection Agency, Duluth, MN.