4. ALGAL TOXICITY TEST

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1. Objective and scope of test method

Algae are included in many hazard assessment schemes as representatives of the aquatic plant community. Algae are ubiquitous in aquatic ecosystems, where they incorporate solar energy into biomass, produce oxygen, function in nutrient cycling, and serve as food for animals. Because of their ecological importance and sensitivity to many substances, especially herbicides and metals, algae are often used in toxicity testing.

The test method described below has been widely used for many years to determine the toxicity of test materials to various species of microalgae. It is derived from a method originally developed in the late 1960's and early 1970's for an "algal assay bottle test" to examine the eutrophication potential of surface waters (U.S. EPA, 1971; Miller et al., 1978). The "bottle test" was subsequently adapted for the purpose of determining toxicity to algae. In this method, which appeared in the mid-1980's (U.S. EPA, 1982; U.S. EPA, 1985; U.S. EPA, 1986), the test material is added to nutrient medium, an inoculum of a single species of algae is added, and the test vessels are incubated under appropriate conditions to examine differences in population growth between treated cultures and controls. This method has been used extensively to determine the toxicity of a variety of test materials, including pesticides (U.S. EPA, 1982; U.S. EPA, 1986; Boutin et al., 1993), industrial chemicals (U.S. EPA, 1985), and effluents (U.S. EPA, 2002). It is sometimes referred to as the "flask method" to distinguish it from scaled-down algal

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tests conducted in vials or microplates. The advantages of this test include the relatively short duration; high replicability and repeatability; minimal requirements for instrumentation and facilities; and the availability of sufficient aqueous sample for analytical confirmation of test concentrations.

2. Summary of test procedure (at a glance)

Organisms of a particular species of microalgae are maintained under static conditions in test vessels containing nutrient medium alone (controls) and nutrient medium to which the test material has been added. In preparation for the test, appropriate volumes of nutrient medium and/or test solution are placed in the test vessels (Erlenmeyer flasks), with replicates for each treatment. Algae are then introduced into the flasks, which are subsequently placed in a growth chamber, which provides standardized light and temperature conditions. Each test vessel is inoculated at an initial population density to provide for growth sufficient to allow accurate quantification without resulting in nutrient or carbon dioxide limitation under the test conditions. Data on population growth during the test are obtained on a daily basis for 96 hours. The results of the test are expressed as the 96-h IC50, based upon final population density and the average specific growth rate. The NOEC (no observed effect concentration) should also be determined. Test results are usually based upon measured test concentrations. Unlike scaled-down test methods, the flask method employs enough test solution for most chemical analytical procedures. The test method is summarized in Table 1.

3. Overview of applications of the algal toxicity test

The flask-based method is the basis of toxicity test methods published by numerous organizations, including the U.S. Environmental Protection Agency (U.S. EPA, 1971; U.S. EPA, 1974; Miller et al., 1978; U.S. EPA, 1978; U.S. EPA, 1982; U.S. EPA, 1985; U.S. EPA, 1986; U.S. EPA, 1996; U.S. EPA, 2002), the Organization for Economic Co-operation and Development (OECD, 1984), and the American Society for Testing and Materials (ASTM, 2003a). These organizations periodically revise their standardized methods, and some changes are anticipated to the cited documents. However, the procedures discussed below reflect the basic test principles that have been in use for over 25 years for a wide variety of toxicity assessment and regulatory purposes. The specific procedures described in this chapter most closely reflect current U.S. EPA approaches to conducting algal toxicity tests with pesticides (under the U.S. Federal Insecticide, Fungicide and Rodenticide Act) and industrial chemicals (under the U.S. Toxic Substances Control Act). For a description of similar Agency methods using algae to determine the toxicity of effluents and receiving waters, refer to U.S. EPA, 2002.

Table 1. Rapid summary of test procedure.

Test type	Static
Test duration	96 hours
Test matrix	Synthetic growth medium appropriate for the test species
Temperature	24°C for *P. subcapitata* and *N. pelliculosa*; 20°C for *S. costatum*
Light quality	Cool-white fluorescent
Light intensity	60 $\mu mol.m^{-2}.s^{-1}$
Photoperiod	Continuous light for *P. subcapitata* and *N. pelliculosa*. 14 h light:10 h dark for *S. costatum*
Shaking	Continuous at 100 oscillations/minute for *P. subcapitata* and *N. pelliculosa*. Manual, once or twice daily, for *S. costatum*
Salinity (for saltwater species)	30 ± 5 ppt (for *S. costatum*)
Test vessel size	125 - 500 mL Erlenmeyer flasks
Test solution volume	≤ 50% of the test vessel volume
Age of inoculum	From logarithmically-growing stock cultures (typically 3 - 7 days old)
Inoculum concentration	10 000 cells/mL for *P. subcapitata* and *S. costatum*. At least 10 000 cells/mL for other species. Inoculum volume < 2 mL
Number of replicates	Four test vessels per concentration (recommended minimum)
Test concentrations	Unless performing a limit test (Section 8.3), a minimum of 5 test concentrations plus appropriate controls
Test concentration preparation	Aqueous solutions prepared by adding test material to synthetic nutrient medium, directly or via carrier
Measurement endpoints	IC50 based upon final population density (yield) and average specific growth rate; NOEC should be observed

4. Advantages of the algal toxicity test

The flask-based method has been in widespread use for many years and has stood "the test of time". It requires only simple equipment that is common in most laboratories, and technicians need minimal training in its use. It employs ecologically relevant organisms that are at the base of the food chain. The test duration is short, although it is inappropriate to term the test an "acute" test, since most test species will undergo several population doublings during the 96-hour exposure period. The algal test thus has an advantage over tests with organisms such as fish and invertebrates, because it measures a population-level response. The basic flask method has been adapted for use with a variety of sample types (including effluents) and test organisms (from cyanobacteria to diatoms). One distinct advantage of the flask method is that it provides a sufficient amount of test solution to allow analytical confirmation of test concentrations, which is often not possible with scaled-down test methods.

5. Test species

Species of algae recommended as test organisms are the freshwater green alga *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* and also as *Raphidocelis subcapitata*); the marine diatom, *Skeletonema costatum*; and the freshwater diatom, *Navicula pelliculosa*. Additional species that have been used include the freshwater green alga *Scenedesmus subspicatus* (recently renamed *Desmodesmus subspicatus*), the marine diatom *Thalassiosira pseudonana,* the marine golden-brown alga *Phaeodactylum tricornutum,* and the marine dinoflagellate *Dunaliella tertiolecta.* Other species, formerly classified as blue-green algae but currently considered cyanobacteria (*Anabaena flos-aquae* and *Microcystis aeruginosa*) can also be tested using these procedures but with a reduced light intensity (see ASTM, 2003a). Additional potential test species are listed by Boutin et al. (1993). The recommended species have been used successfully and have been demonstrated to be sensitive to a variety of test substances. The responses of algal species vary and there is no single "most sensitive" species. Therefore, testing of several species may be needed. For pesticide registration, the U.S. EPA requires (depending upon the use pattern of the pesticide) testing with four species: *P. subcapitata*, *A. flos-aquae*, *S. costatum* and a freshwater diatom such as *N. pelliculosa*.

6. Culture/maintenance of organism in the laboratory

6.1 SOURCE, AGE AND CONDITION

Algae to be used in toxicity tests may be initially obtained from commercial sources and subsequently cultured using sterile technique. Commercial sources include the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, 20852 and the University of Texas Algal Collection, Botany Department, Austin, TX,

78712. Upon receipt of an algal culture not previously maintained in a facility, a period of six weeks culturing is recommended to establish the ability to successfully maintain a healthy, reproducibly-growing culture. Information on culturing algae can be found in the references listed in the ASTM Guide E-1218 (ASTM, 2003a). Aseptic stock transfer should be performed on a regular schedule (*e.g.*, once or twice weekly) to maintain a supply of cells in or near the logarithmic growth phase. Long-term maintenance of cultures on a solid medium containing 1% agar in sterile Petri plates or test tubes may be desirable. However, the algal inoculum used to initiate toxicity testing must be from a liquid culture shown to be actively growing (*i.e.,* capable of logarithmic growth within the test period) in at least two subcultures lasting 7 days each prior to the start of the definitive test.

6.2 APPARATUS AND FACILITIES

Normal laboratory equipment and especially the following are necessary:

- Equipment for determination of test conditions (*e.g.*, pH meter and light meter).
- Containers for culturing and testing algae. Erlenmeyer flasks should be used as test vessels. The flasks may be of any volume between 125 and 500 mL as long as the same size is used throughout testing and the test solution volume does not exceed 50 percent of the flask volume. To permit gas exchange but prevent contamination, the flasks should be covered with foam plugs, stainless steel caps, 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). All test vessels and covers in a test must be identical.
- A growth chamber or a controlled environment room that can hold the test vessels and will maintain the air temperature, lighting intensity, and photoperiod specified in this test guideline. If necessary for the species, a mechanism for continuously shaking the test vessels.
- Apparatus for preparing sterile nutrient media.
- Apparatus for sterilizing glassware and maintaining aseptic technique during culturing and testing.
- Microscope capable of 100 to 400 X magnification.
- Apparatus for enumerating algae, *e.g.*, hemacytometer, plankton counting chamber, or electronic particle counter. An alternative method to performing cell counts is to determine the chlorophyll *a* concentration through spectrophotometric or fluorometric methods.
- 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 not contain substances that can be leached or dissolved into aqueous solutions in quantities that can affect the test results. Construction materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test materials.

6.3 CLEANING AND STERILIZATION OF GLASSWARE

New test vessels may contain substances which inhibit growth of algae. They are therefore to be cleaned thoroughly and used several times to culture algae before being used in toxicity testing. All reusable glassware employed in algal culturing or testing is to be cleaned and sterilized prior to use. Wash glassware using a non-phosphate detergent and a stiff bristle brush to remove residues. This is followed by thorough rinsing with water, a rinse with a water-miscible solvent (such as acetone), additional rinsing with water, a rinse with acid (such as 10% hydrochloric acid), and at least two final rinses with reagent grade water. These procedures are generally suitable to remove test material residues from previous toxicity testing, but additional procedures may be required depending upon the nature of the test material.

Glassware may be dried in an oven at 50 to 100ºC, capped with flask closures or covered loosely with foil, and sterilized by autoclaving for 20 minutes at 121ºC and 1.1 kg/cm^2.

6.4 PREPARATION OF NUTRIENT MEDIA

Water used for preparation of nutrient medium should be of reagent quality (*e.g.,* ASTM Type I water). Freshwater algal nutrient medium (AAP or "Algal Assay Procedure" medium, as described by Miller et al., 1978) is prepared by adding specified amounts of reagent-grade chemicals to reagent water. Marine algal nutrient medium is prepared by adding reagent grade chemicals to synthetic salt water (see Walsh and Alexander, 1980) or to filtered natural salt water, or by preparing a complete saltwater medium. Salinity for saltwater medium should be 30 ± 5 ppt.

Formulation and sterilization of nutrient medium used for algal culture and preparation of test solutions should conform to those currently recommended by ASTM for freshwater and marine algal toxicity tests (see Tables 2 and 3). Chelating agents (*e.g*. EDTA) are included in the nutrient medium for optimum cell growth. Nutrient medium should be freshly prepared for algal testing or may be stored under refrigeration for several weeks prior to use. Nutrient medium should be sterilized by autoclaving or filtering (0.22 µm filter). At the start of the test, the pH of the nutrient medium should be 7.5 ± 0.1 for freshwater algal medium and 8.0 ± 0.1 for marine algal medium. The pH may be adjusted prior to addition of the test material with 0.1N or 1N sodium hydroxide or hydrochloric acid.

Table 2. Preparation of medium for freshwater algae.

This medium (often referred to as AAP medium) is prepared by adding 1 mL of each macronutrient stock solution and 1 mL of the micronutrient stock solution listed below to approximately 900 mL reagent grade water and then diluting to 1 L.

Each of the six macronutrient stock solutions is prepared by dissolving each of the following chemicals into 500 mL of reagent grade water:

1) $NaNO_3$ — 12.750 g
2) $MgCL_2{\cdot}6H_2O$ — 6.082 g
3) $CaCl_2{\cdot}2H_2O$ — 2.205 g
4) $MgSO_4{\cdot}7H_2O$ — 7.350 g
5) K_2HPO_4 — 0.522 g
6) $NaHCO_3$ — 7.500 g

For diatom species only, add $Na_2SiO_3{\cdot}9H_2O$ as another macronutrient. May be added directly (202.4 mg) or by way of a stock solution to give a final concentration of 20 mg/L Si in medium.

The micronutrient stock solution is prepared by dissolving the following chemicals into 500 mL of reagent water:

H_3BO_3 — 92.760 mg
$MnCl_2{\cdot}4H_2O$ — 207.690 mg
$ZnCl_2$ — 1.635 mg
$FeCl_3{\cdot}6H_2O$ — 79.880 mg
$CoCl_2{\cdot}6H_2O$ — 0.714 mg
$Na_2MoO_4{\cdot}2H_2O$ — 3.630 mg
$CuCl_2{\cdot}2H_2O$ — 0.006 mg. (Typically must be prepared by serial dilution).
$Na_2EDTA{\cdot}2H_2O$ — 150 mg. [Disodium (Ethylenedinitrilo) tetraacetate].
($Na_2SeO_4{\cdot}5H_2O$ — 0.005 mg. Used only in medium for stock cultures of diatom species)

Adjust pH to 7.5 ± 0.1 with 0.1 *N* or 1.0 *N* NaOH or HCl.

Filter all media into a sterile container through a 0.22 µm membrane filter if a particle counter is to be later used for enumerating algal cells otherwise through a 0.45-µm filter. Store medium in the dark at approximately 4°C until use.

Table 3. Preparation of medium for saltwater algae.

The Micronutrient Mix is prepared by adding the specified amount of chemicals in the order listed below to 900 mL reagent water and diluting to 1 L.

Micronutrient Mix:
$FeCl_3 \cdot H_2O$ — 0.048 g
$MnCl_2 \cdot 4H_2O$ — 0.144 g
$ZnSO_4 \cdot 7H_2O$ — 0.045 g
$CuSO_4 \cdot 5H_2O$ — 0.157 mg
$CoCl_2 \cdot 6H_2O$ — 0.404 mg
H_3BO_3 — 1.140 g
$Na_2EDTA.2H_2O$ — 1.0 g

The Minor Salt Mix is prepared by adding the specified amounts of the chemicals listed below to 900 mL reagent water and diluting to 1 L.

Minor Salt Mix:
K_3PO_4 — 0.3 g
$NaNO_3$ — 5.0 g
$NaSiO_3 \cdot 9H_2O$ — 2.0 g

The Vitamin Mix is prepared by adding the specified amount of chemicals in the order listed below to 900 mL reagent water and diluting to 1 L.

Vitamin Mix:
Thiamine Hydrochloride — 500 mg
Biotin — 1 mg
B_{12} — 1.0 mg

The stock solutions are added to a sterile recipient containing either natural salt water that has been filtered through a 0.22 µm membrane filter or reconstituted salt water. Add the amounts given below to prepare medium used for toxicity testing. Add twice the amounts given to prepare medium for use in maintenance of stock cultures.

Add 15 mL of Micronutrient Mix/L of medium
Add 10 mL of Minor Salt Mix/L of medium
Add 0.5 mL of Vitamin Mix/L of medium. (Add 1 mL of vitamin mix if *Thalassiosira* is used).

Adjust pH to 8.0 ± 0.1 with 0.1 *N* or 1.0 *N* NaOH or HCl. Store medium in the dark at approximately 4°C until use.

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7. Preparation of test species for toxicity testing

The cultures used as the source of inoculum should be maintained under the same conditions as used for testing. The algal inoculum to begin the toxicity test should be from logarithmically-growing stock cultures (typically 3 to 7 days old). All algae used for a particular test should be from the same source and the same stock culture. Also, the clone of all species should be specified. Test algae must not have been used in a previous test, either in a treatment or a control. A culture should not be used for starting a test if it is not in logarithmic growth phase, if microscopic examination at 400 X shows contamination by fungi or other algae, or if the health of the culture is doubtful in any respect.

Each test vessel should be inoculated at an initial population density to allow sufficient growth under the test conditions without resulting in nutrient or carbon dioxide limitation. The primary criterion for the initial cell concentration is that accurate estimates of population density can be obtained with the chosen method of measurement during the test. For *P. subcapitata* and *S. costatum*, the initial cell concentration should be 10 000 cells/mL. Higher concentrations may be necessary for other species, but the upper limit should be no more than 100 000 cells/mL. It is not usually necessary to concentrate the algal cells as part of inoculum preparation. The volume of inoculum to be added to each test vessel is calculated based upon the cell concentration in the stock culture, the volume in the test vessel, and the desired initial cell concentration. It is important to maintain aseptic technique in all culturing and testing procedures.

8. Testing procedure

8.1 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 material, *e.g*., 0.1, 1.0, 10, 100 mg/L, etc. (Note that for effluents, range-finding tests may not be practical due to limitations on holding times of samples). In a range-finding test, no replicates are required and nominal concentrations of the test material are acceptable.

8.2 DEFINITIVE TEST

The goal of the definitive test is to determine concentration-response curves and IC50 values (with 95 percent confidence intervals and standard error) for algal population growth for each species tested. In addition, the slopes of the concentration-response curves, the associated standard errors and the 95% confidence intervals of the slopes should be determined. For this determination, a minimum of five concentrations of the test material, plus appropriate controls, are required. The range of concentrations tested should bracket the expected IC50 value. Analytical confirmation of test concentrations should be performed using an

acceptable validated analytical method. At the end of the exposure period, algistatic and algicidal effects can be determined as described in Section 9.4.

8.3 LIMIT TEST

In some situations, it is only necessary to ascertain that the IC50 is above a certain limit. A limit test has also been referred to as a Tier I test or Maximum Challenge Concentration test. In a limit test, at least three replicate test vessels are exposed to a single "limit concentration," with the same number of test vessels containing the appropriate control solution(s). If the IC50 is greater than the limit concentration, multiple-concentration definitive testing may be waived. Acceptable limit tests must meet all the requirements for acceptable multi-concentration definitive tests, with the exception of the number of test concentrations and endpoint determinations. Acceptable limit tests require analytical confirmation of the limit concentration.

8.4 PREPARATION OF TEST MATERIAL

8.4.1 Basic information

Basic information about the test material should be known prior to testing. This includes the following: chemical name; CAS number; molecular structure; source; lot or batch number; purity and/or percent active ingredient (a.i.); identities and concentrations of major ingredients and major impurities; date of most recent assay and expiration date for sample. In addition, it is important to know the appropriate storage and handling conditions for the test material to protect the integrity of the test material and the solubility and stability of the test material under test conditions. Physico-chemical properties of the test material can affect the design and interpretation of the test, and should be considered carefully. These include: solubility in water and various solvents; vapor pressure; hydrolysis at various pH, etc.

8.4.2 Preparation of stock solution

In some cases, test solutions are prepared by adding the test material directly to the growth medium on a weight/volume or volume/volume basis. More often, a stock solution of the test material is prepared and aliquots of the stock solution or secondary stock solutions are added to the growth medium. The preferred practice is to make a bulk preparation of each test solution and distribute portions to each replicate test vessel. Samples are taken from the bulk preparations for analytical confirmation of initial test concentrations.

The preferred choice for preparation of the stock solution is to use reagent water (deionized, distilled or reverse osmosis water), providing the test material can be dissolved in water and does not readily hydrolyze, and providing that the amount of stock solution added to the growth medium will be less than 10% of the total volume (in order to avoid changes in the growth medium). To avoid alterations in the growth medium (*e.g.*, unacceptable change in salinity or in concentration of nutrients), the stock solution may also be prepared in growth medium.

If the test material cannot be dissolved in reagent water or growth medium, carriers are often used. If a carrier, *i.e.*, a solvent and/or a dispersant, is absolutely

necessary to dissolve the test material, the amount used should not exceed the minimum volume necessary to dissolve or suspend the test material in the growth medium. If the test material is a mixture, formulation or commercial product, none of the ingredients is considered a carrier unless an extra amount is used to prepare the stock solution. The preferred solvent for algal toxicity tests is N,N-dimethylformamide, as solvents such as acetone can cause stimulation of bacterial growth (Hughes and Vilkas, 1983). The concentration of solvent should preferably be the same in all test treatments and should not exceed 0.1 mL/L.

Solvent use should be avoided if possible. If a carrier is employed, a carrier control must be included in the test, in addition to the growth medium control. The selected carrier should not affect the test organisms at the concentration used. The carrier (solvent) control must be prepared from the same batch of solvent as that used to prepare the test treatment solutions.

The pH may be adjusted in stock solutions to match that of the medium if pH change does not affect the stability of the test material in the stock solution or test solution. Hydrochloric acid and sodium hydroxide may be used for this adjustment if warranted. The pH should generally not be adjusted after the addition of the test material or stock solution into the test medium. If the test material is highly acidic and reduces the pH of the test solution below 5.0 at the first measurement, or is highly basic and increases the pH of the test solution similarly, appropriate adjustments should be considered, and the test solution measured for pH on each day of the test. If the pH of the test solutions is altered, a concurrent test without pH adjustment of the test solutions is recommended.

8.4.3 Test concentrations

A toxicity test designed to allow calculation of a regression-based estimate such as an IC50 usually consists of one or more control treatments and at least five test solution concentrations. The test solution concentrations are usually selected in a geometric series in which the ratio is between 1.5 and 3.2. The selection of test concentrations depends upon the expected slope of the dose-response curve, which can be determined based upon the results of the range-finding test. Some methods for calculating the IC50 require that the test concentrations be equally spaced, while some methods do not.

8.5 ENVIRONMENTAL CONDITIONS

The test temperature is 24°C for *P. subcapitata* and *N. pelliculosa*, and 20°C for *S. costatum*. Excursions from the test temperature should be no greater than ± 2°C. Test vessels containing *P. subcapitata* and *N. pelliculosa* should be illuminated continuously; those containing *S. costatum* are to be provided a 14 h light:10 h dark photoperiod. Cool-white fluorescent lights providing 60 $\mu mol.m^{-2}s^{-1}$ should be used (for cool-white fluorescent lighting, this is approximately equivalent to 4300 lux). A PAR (photosynthetically active radiation) sensor should be used to measure light quality and measurements should be made at each test vessel position at the approximate level of the test solution. The light intensity should not vary more than ± 15% from the selected light intensity at any test vessel position in the incubator or

growth chamber. Additional information on the use of lighting in plant toxicity tests can be found in ASTM E-1733 (ASTM, 2003b).

Stock algal cultures of *P. subcapitata* and *N. pelliculosa* should be shaken on a rotary shaking apparatus. Test vessels containing these species should also be placed on a rotary shaking apparatus and oscillated at approximately 100 cycles/min during testing. The rate of oscillation should be determined at the beginning of the test or at least once daily during testing if the shaking rate is changed or changes. Culture and test vessels containing *S. costatum* should be shaken by hand once or twice daily. If clumping of cells is not experienced, *S. costatum* may be continuously shaken at approximately 60 cycles/min.

9. Observations/measurements and endpoint determinations

9.1 MEASUREMENT OF TEST MATERIAL

Analytical confirmation of test concentrations should be performed at test initiation and at test termination. The analytical method used to measure the amount of test material in a sample should be validated before beginning the test. Samples for analysis of initial test concentrations should be collected from the bulk preparations used to begin the test. 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 is determined after all algal cells have been removed, either by centrifugation or filtration. The effect of centrifugation or filtration upon recovery of the test material should be determined during method validation. As an additional procedure, the concentration of test material associated with the algae alone may be determined, if desired. To do this, separate and concentrate the algal cells from the test solution by centrifuging or filtering the remaining pooled sample and measure the test material concentration in the cell concentrate.

Observations on test material solubility should be recorded. The appearance of surface slicks, precipitates, or material adhering to the sides of the test vessels should also be recorded.

9.2 MEASUREMENT OF ENVIRONMENTAL CONDITIONS

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 vessels are placed in an environmental chamber or incubator, the air temperature is more likely to fluctuate than the water temperature.

The pH in control and test solutions should be measured at the beginning and end of the test. It can be measured in the bulk test solutions at test initiation and in

samples of pooled replicates of each test treatment at test termination (provided none of the replicates appear to be "outliers" with respect to growth, in which case individual pH measurements should be made).

As testing begins, light intensity (light fluence rate) should be monitored at the approximate level of the test solution at each test chamber position in the growth chamber. Random repositioning of the test vessels on a daily basis during the test is recommended to minimize spatial differences in temperature and lighting.

9.3 BIOLOGICAL OBSERVATIONS

The test is based upon the increase in algal biomass observed in exposed cultures compared to that in the control. Because biomass (*e.g.*, the dry weight of living matter present in a given volume) is difficult to measure accurately, surrogate measures of biomass are typically used in this test. The most common measure is to determine algal population density by counting the number of cells in a given volume. Cell counts in each test vessel should be determined at 24, 48, 72 and 96 hours. Performing cell counts using direct microscopic observation or using an electronic particle counter are both acceptable methods for determining population density. Chlorophyll *a* (measured spectrophotometrically or fluorometrically) or other measurements may also be used. Dry weight, although a direct measure of biomass, is a destructive measure that can only be used at test termination and must be accomplished carefully to obtain accurate results.

Microscopic counting of cells can be performed using a hemacytometer or an inverted microscope with settling chambers. Precision is proportional to the square root of the number of cells counted. For microscopic counting, two samples should be taken from each test vessel and two counts made of each sample. Whenever feasible, at least 400 cells per test vessel should be counted in order to obtain ± 10% accuracy at the 95% confidence level.

An alternative method to enumerate large numbers of cells very rapidly is to use an electronic particle counter. It is recommended that the laboratory develop data demonstrating the correlation between electronic particle counts and microscopic counts for each algal species. Automated particle counting, although the most rapid and sensitive method, has limitations, some related to particle interferences. If the test solution does not have a low background in the particle size range of the test species, masking errors will result. An additional test vessel at each concentration containing test material and growth medium without algae can allow measurement of potential particle interference.

Microscopic observations at test termination should be performed to determine whether the altered growth response between controls and test algae (at the concentrations of test material demonstrating an effect) was due to a change in relative cell numbers, cell sizes, or both. Noting any unusual cell shapes, color differences, differences in chloroplast morphology, flocculations, adherence of algae to test vessels, or aggregation of algal cells is also recommended. While these observations are qualitative and descriptive, they are independent of endpoint calculations. They can be useful, however, in demonstrating additional effects of test materials.

Other measurements that may be useful include determination of mean cell volume, organic carbon content of the cells, and dry weight. These measurements are not routinely required but may provide important information if the test material has an effect upon algal biomass that is not reflected in cell counts.

9.4 DETERMINATION OF ALGISTATIC AND ALGICIDAL EFFECTS

At the end of the 96-hour exposure period, determination of algistatic and algicidal effects may be performed, if desired (Payne and Hall, 1979). If the test material is algicidal, the algae have been killed and the population is unable to recover. If the test material is algistatic, population growth is inhibited in the presence of the test material but resumes once it is removed. In test concentrations where growth is maximally inhibited, algistatic effects may be differentiated from algicidal effects by either of the following two methods.

(1) Add 0.5 mL of a 0.1 percent solution (weight/volume) of Evans blue stain to a 1-mL aliquot of algal suspension from a control vessel and to a 1-mL aliquot of algae from the test vessel having the lowest concentration of test material which completely inhibited algal growth. Complete inhibition of algal growth is demonstrated if the algal population density at 96 hours is approximately the same as the initial population density. If algal growth was not completely inhibited, select an aliquot of algae for staining from the test vessel having the highest concentration of test material where at least some algal growth inhibition has occurred. Wait 10 to 30 min, examine microscopically, and determine the percent of the cells which stain blue (indicating cell mortality). A staining control is to be performed concurrently using heat-killed or formaldehyde-preserved algal cells; 100 percent of these cells should stain blue. This method will work for *S. costatum* (as it was initially developed with this species) and possibly *Navicula* spp., but it may not work with *P. subcapitata*.

(2) Remove 0.5 mL aliquots of test solution containing growth-inhibited algae from each replicate test vessel having the lowest concentration of test material which completely inhibited algal growth. If algal growth was not completely inhibited, select aliquots from the highest concentration of test material indicating algal growth inhibition. Combine these aliquots into a new test vessel and add a sufficient volume of fresh nutrient medium to dilute the test material to a concentration which does not affect growth (using the original test vessel size and solution volume is generally appropriate). Aliquots from the control test vessels are also transferred to clean medium. Incubate these subcultures under the environmental conditions used during the exposure period for up to 9 days, and observe periodically (*e.g.*, every other day) for algal growth to determine if the algistatic effect noted after the 96-h exposure is reversible. This subculture test may be discontinued as soon as growth occurs.

9.5 TREATMENT OF RESULTS

Algal population density is the biomass measurement normally used to evaluate the inhibitory and stimulatory effects of the test material. Two response variables are calculated: final population density, also referred to as yield, and average specific growth rate. The IC50 value is determined (with 95 percent confidence interval and standard error, as well as slope of the concentration-response curve, standard error, and 95 percent confidence interval) for each of these response variables. The NOEC and LOEC should also be determined. The calculation of measurement endpoints at 72 h, in addition to 96 h, is desirable, provided growth is sufficient for analysis at these earlier time periods.

9.5.1 Use of measured concentrations

Results are expressed based upon measured concentrations of the test material, if available. If analytical verification of test concentrations has not been performed, the nominal values are used.

One of the advantages of this test design over scaled-down tests is that sufficient sample volume is usually available to measure the test concentrations at the beginning and end of the test. Thus, the flask-based test is the method of choice where analytical confirmation is needed. It is not uncommon, however, for test concentrations to decline during the exposure period, usually due to inherent properties of the test material, although uptake and adsorption by algal cells can also occur. Analysis of the concentration in a “blank” test vessel (prepared and incubated as the other replicates for a particular concentration, but not inoculated with algae) can shed light on these phenomena. If the test material concentrations decline during the exposure period, it may be possible to determine the rate of decline and use this to calculate the actual exposure concentrations. Otherwise, the mean of the initial and final measured concentrations is used as an approximation. Alternatively, if concentrations decline by less than an amount set by the precision of the analytical method (typically about 20%), the initial concentrations may be used. Because this test is a static toxicity test, there is little that can be done to maintain test concentrations during the exposure period. Conducting flow-through and renewal exposure procedures with microalgae are currently impractical, which can be a disadvantage to this and other phytotoxicity tests.

9.5.2 Final population density

Final population density at test termination (96 h) for each test vessel, or more correctly, yield, is used to calculate the IC50. To correctly represent yield, the initial population density values should be subtracted from the final population density values for each test vessel. Since the initial values are extremely small relative to the final values, this correction has a small impact upon the test results but is nonetheless recommended. Population densities at the end of 24, 48 and 72 hours can also be used to calculate IC50s for those time periods, if desired, and if growth is sufficient.

9.5.3 Average specific growth rate

Average specific growth rate is also used to calculate the IC50. It represents the growth rate calculated over the entire test period. In addition, the specific growth rate during the course of the test (days 0-1, 1-2, 2-3, etc.), also called the section-by-section growth rate, should be calculated to assess effects of the test material, such as an increased lag phase, occurring during the exposure period. Substantial differences between the section-by-section growth rates and the average growth rates indicate deviation from theoretical exponential growth and that close examination of these data are warranted. In this instance, the recommended approach is to compare specific growth rates from exposed cultures during the time period of maximum inhibition to those for controls during the same period. The same time interval should be used for each test vessel in all treatments. The growth rate for each test vessel over the selected time interval is calculated as follows:

$$\mu = \frac{lnN_2 - lnN_1}{t_2 - t_1} \tag{1}$$

where:
μ = growth rate, in day^{-1}
N_1 = population density at the beginning of the selected time interval
N_2 = population density at the end of the selected time interval
t_2 = time at end of interval (in days)
t_1 = time at start of interval (in days).

9.5.4 Calculation of IC50

The IC50 and 95% confidence interval are determined using an appropriate statistical model to establish the concentration-response curve for the response variables. The values for each test vessel, not the mean for each concentration, should be used as the response variable in fitting the model.

Statistical procedures for modeling continuous toxicity data are available and should be used (Bruce and Versteeg, 1992; Nyholm et al., 1992; VanEwijk and Hoekstra, 1993). Regarding terminology, the term ICx is often used for non-quantal endpoints, rather than ECx.

Percent inhibition (%I) at each test concentration is calculated as follows:

$$\%I = \frac{C-X}{C} \times 100 \tag{2}$$

where: C = the average value of the response variable in the control test vessels and X = the average value of the response variable in the test treatment. Stimulation is reported as negative percent inhibition.

9.5.5 Calculation of NOEC and LOEC

Hypothesis testing procedures can be used to determine the NOEC and LOEC for each of the measured response variables. Assumptions of statistical procedures should be examined and verified as met prior to their use. Results of hypothesis tests

should be reported along with some measure of the sensitivity of the test (either the minimum significant difference or the percent change from the control that this minimum difference represents).

9.6 TEST ACCEPTABILITY

Validity criteria for the test include acceptable growth in the controls and acceptable variation between control replicates. During 96 hours, cell counts in the controls should increase by a factor of at least 100 times for *P. subcapitata* and a factor of at least 30 times for *S. costatum*. The appropriate increase within 96 hours for *N. pelliculosa* has not been determined at this time. For any algal species, the coefficient of variation for yield in the control should be calculated and should generally be less than 20%. For growth rate, which is a logarithmically-transformed variable, the coefficient of variation should be substantially less than 20% (*e.g.*, < 12%).

9.7 REPORTING

The reported results of the test should include the following:

- Test facility, dates and personnel.
- Identification of test material and purity.
- Description of the preparation of the synthetic growth media used, the concentrations of all media constituents, and the initial pH.
- Methods of stock solution and test solution preparation and the concentrations of test material and solvent, if applicable, used in definitive testing.
- Detailed information about the test organisms, including the scientific name, method of verification, strain, and source. Information about the culture practices and conditions. Description of preparation of inoculum used to begin test.
- A description of the growth chamber and test vessels, the volumes of solution in the test vessels, the way the test was begun (*e.g.*, conditioning, test material additions, etc.), the number of replicates, the temperature, the lighting, and method of incubation, oscillation rates, and type of apparatus. Specific modifications in test procedures due to using species other than those recommended must be noted.
- The concentration of the test material in the control(s) and in each treatment at the beginning and end of the test and the pH of the solutions at the beginning and end of the test.
- The number of algal cells per milliliter in each test vessel (or other biomass surrogate data) and the method used to derive these values at the beginning, at 24, 48, and 72 h, and at the end of the test; calculated mean values with standard deviation; the percentage of inhibition or stimulation of growth

relative to controls (based upon means); and other adverse effects in the control and in each treatment.

- The 96-h IC50 values, and when sufficient data have been generated, the 24-, 48-, and 72-h IC50s and 95 percent confidence limits. The IC50 should be determined based upon final population density (yield) and average specific growth rate. The slopes of the concentration-response curves, associated standard errors and the 95% confidence intervals of the slope should be reported as well. NOEC/LOEC values should also be reported.
- Methods of statistical analysis, including software used, should be described.
- Methods used in the analysis of concentrations of test material should be described. The accuracy of the method, method detection limit, and limit of quantification should be given.
- Microscopic appearance of algae, size or color changes, and any other observed effect.
- If determined, report the algistatic and algicidal concentrations.
- For a limit test, report the percent effect upon the measured response variables at the tested concentration.
- Any protocol deviations or occurrences which may have influenced the final results of the test.

10. Factors capable of influencing algal growth and test results

Test solutions that are highly colored or opaque can reduce or prevent light transmission, affecting algal photosynthesis due to a physical effect rather than a toxicological effect. Test materials that are highly volatile can escape from the test system, since the flask stoppers permit gas exchange (and thus allow photosynthesis). It is possible to modify the test design to accommodate highly volatile materials by adding supplemental carbon and eliminating the head space (*e.g.*, using a BOD bottle), but such procedures are not part of the typical method. Some test materials (*e.g.*, some anionic polymers) cause chelation of the trace nutrients needed for algal growth. Since the nutrient medium for freshwater algae has a low hardness, growth inhibition can be observed in these circumstances and interpreted as toxicity. However, when sufficient calcium (as divalent cation) is added to satisfy the ionic charge of the polymer, toxicity to algae is mitigated (Nabholz et al., 1993).

11. Application of the algal toxicity test in a case study

Several algal species were used to evaluate the toxicity of the herbicide atrazine in a study that reported the IC50, NOEC, and algistatic and algicidal endpoints (Hughes et al., 1988). This study used a 5-day exposure period, and there were some minor

differences in the methods relative to those described in this chapter. However, the approach taken and the comparison of the test endpoints are illustrative of the principles of the flask-based algal toxicity test. The test species included the freshwater diatom *Navicula pelliculosa*, the marine flagellate *Dunaliella tertiolecta*, and the cyanobacteria *Anabaena flos-aquae*. The results are presented in Table 4.

Table 4. Effects of atrazine on three species in the algal toxicity test.

Species	*NOEC mg/L*	*IC50[1] mg/L*	*Algistatic concentration[1] mg/L*	*Algicidal concentration mg/L*
Anabaena flos-aquae	< 0.1	0.23 (0.12 – 0.38	4.97 (2.39 – 14.2)	> 3.2
Dunaliella tertiolecta	< 0.1	0.17 (0.11 – 0.26)	1.45 (0.44 – 6.72)	3.2
Navicula pelliculosa	< 0.1	0.06 (0.002 – 0.21)	1.71 (0.40 – 13.2)	> 3.2

[1] The 95% confidence limits are given in parentheses.

Each species was significantly affected by the lowest test concentration of atrazine, thus the NOEC was below 0.1 mg/L. The IC50 values were calculated based upon final population density, and ranged from 0.06 mg/L for *N. pelliculosa* to 0.23 mg/L for *A. flos-aquae*. (IC50 values based upon average specific growth rate were not determined). The algistatic concentration was determined as the concentration of test material at which the population density on day 5 was the same as the initial population density. This value ranged from 1.45 mg/L for *D. tertiolecta* to 4.97 mg/L for *A. flos-aquae*. *D. tertiolecta* was unable to recover from exposure to a concentration of 3.2 mg/L atrazine, while the other species did recover, indicating that atrazine was not algicidal to *N. pelliculosa* and *A. flos-aquae*. Atrazine prevented photosynthesis, but since all of the algal cells were not killed, the population of these two species was able to recover in the absence of atrazine. This can be useful information for a variety of risk assessment applications, especially if the test substance is expected to have a short duration of use or limited stability in the environment. Although a continuous exposure to an algistatic concentration of a test substance would cause complete inhibition of growth, in the absence of continuous input, recovery of the algal population would be expected as the test substance degrades. Due to the additional time and effort required to determine algistatic and algicidal effects, however, the use of the IC50 is an acceptable and conservative way to express toxicity to algae. It should be emphasized that an IC50 for algae represents a population effect and that it is not analogous to similar endpoints (*e.g.*, EC50 or LC50) for aquatic animals.

12. Miscellaneous test information

Algal species vary in their sensitivity and no single species is the most sensitive to all toxicants (Blanck et al., 1984; Peterson et al., 1993). For this reason, it is recommended to test several species to appropriately define potential hazard (Lewis, 1990; Swanson et al., 1991). The flask-based algal toxicity test is adaptable to use with various species, although changes in the initial inoculum concentration, test duration, incubation conditions and medium may be necessary.

13. Conclusions

The algal toxicity test described in this chapter has been widely used for at least 25 years to determine the toxicity of a variety of test substances to microalgae. The method has undergone standardization by groups including ASTM and OECD. It is a practical means to evaluate toxicity to organisms that are considered to be the basis of the food web in most aquatic systems. However, it is unlikely that algae can reliably serve as surrogates for higher aquatic plants, and additional developmental work is needed on test methods with submersed and emergent aquatic macrophytes to examine the relative sensitivity of all of these organisms. Assessing the response of aquatic plants is critical to the risk assessment process, particularly for chemicals such as herbicides. For example, U.S. EPA's new draft ambient water quality criterion for atrazine was derived based upon changes in aquatic plant community structure, as this was the most sensitive response observed (U.S. EPA, 2003).

The algal toxicity test examines the response of only one species at a time and thus does not consider interactions within the algal community, which can be an important influence upon the overall productivity of the aquatic ecosystem. New multi-species test procedures (Franklin et al., 2004) show promise in elucidating these types of interactions; however, they require the use of sophisticated equipment not currently in routine use in most laboratories.

Although the procedures for conducting the algal toxicity test are straightforward, there is room for improvement in understanding, interpreting and using the results in risk assessment. Probably due to its long history as one of the basic tests in a "tiered" risk assessment, an algal IC50 is too often equated with a measure of mortality in an acute exposure for an aquatic fish or invertebrate. Research to establish the linkage of laboratory tests with microalgae to responses in field situations would advance the utility of algal toxicity test data. As pointed out by Lewis (1990), the significance of reductions in algal growth observed in a laboratory test must be interpreted in light of ecological factors such as adaptation and compensation to improve the utility of laboratory results in risk assessment.

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References

ASTM (American Society for Testing and Materials) (2003a) Standard guide for conducting 96-h toxicity tests with microalgae, in *Annual Book of ASTM Standards*, Vol. 11.05, ASTM E 1218-97a. West Conshohocken, PA., Current edition approved October 10, 1997.

ASTM (American Society for Testing and Materials) (2003b) Standard guide for use of lighting in laboratory testing, in *Annual Book of ASTM Standards*, Vol. 11.05, ASTM E 1733 -95. West Conshohocken, PA., Current edition approved September 10, 1995.

Boutin, C., Freemark, K.E. and Keddy, C.J. (1993) Proposed Guideline For Registration Of Chemical Pesticides: Nontarget plant testing and evaluation, Tech. Rpt. Series No. 145, Canadian Wildlife Service, Environment Canada.

Blanck, H., Wallin G. and Wangberg, S. (1984) Species-dependent variation in algal sensitivity to chemical compounds, *Ecotoxicology and Environmental Safety* **8**, 339-351.

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

Franklin, N.M, Stauber, J.L. and Lim, R.P. (2004) Development of multispecies algal bioassays using flow cytometry, *Environmental Toxicology and Chemistry* **23**, 1452 – 1462.

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, in W.J. Adams, G.A. Chapman, and W.G. Landis, (eds.), *Aquatic Toxicology and Hazard Assessment: 10th Volume*, ASTM STP 97, American Society for Testing and Materials, Philadelphia, pp. 531 - 547.

Hughes, J.S. and Vilkas, A.G. (1983) The Toxicity of N,N-dimethylformamide used as a solvent in toxicity tests with the green alga, *Selenastrum capricornutum*, *Bulletin of Environmental Contamination and Toxicology* **31**, 98-104.

Lewis, M.A. (1990) Are laboratory-derived toxicity data for freshwater algae worth the effort? *Environmental Toxicology and Chemistry* **9**, 1279-1284.

Miller, W.E., Greene, J.C. and Shiroyama, T. (1978) The *Selenastrum capricornutum* Printz Algal Assay Bottle Test: Experimental Design, Application, and Data Interpretation Protocol, EPA-600/9-78-018, Corvallis, OR.

Nabholz, V.N, Miller P. and Zeeman, M. (1993) Environmental risk assessment of new chemicals under the Toxic Substances Control Act (TSCA) Section Five, in W.G. Landis, J.S. Hughes, and M.A. Lewis (eds.), *Environmental Toxicology and Risk Assessment.,* ASTM STP 1179, American Society for Testing and Materials, Philadelphia, PA., pp. 40-55.

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

OECD (Organization for Economic Co-operation and Development) (1984) OECD Guidelines for Testing of Chemicals, TG 201, Alga, Growth Inhibition Test.

Payne, A.G. and Hall, R.H. (1979) A method for measuring algal toxicity and its application to the safety assessment of new chemicals, in L.L. Marking and R.A. Kimerle (eds.), *Aquatic Toxicology*, ASTM STM 667, American Society for Testing and Materials, Philadelphia, PA., pp. 171-180.

Peterson, H.G., Boutin, C., Freemark, K., Martin, P.A., Ruecker N.J. and Moody, M.J. (1993) Aquatic phytotoxicity of 23 pesticides applied at expected environmental concentrations, *Aquatic Toxicology* **28**, 275-292.

Swanson, S.M., Rickard, C.R., Freemark K.E. and MacQuarrie, P. (1991) Testing for pesticide toxicity to aquatic plants: Recommendations for test species, in J.W. Gorsuch, W.R. Lower, M.A. Lewis and W. Wang, (eds.), *Plants for Toxicity Assessment*, Vol. 2, STP 1115, American Society for Testing and Materials, Philadelphia, PA., pp. 77-97.

U.S. EPA (U.S. Environmental Protection Agency) (1971) Algal Assay Procedure Bottle Test. National Eutrophication Research Program, Office of Research and Development, Environmental Research Division, Western Ecology Division, Corvallis, OR., 82 pp.

U.S. EPA (U.S. Environmental Protection Agency) (1974) Marine Algal Assay Procedure: Bottle Test, Eutrophication and Lake Restoration Branch, Pacific Northwest Environmental Research Center, Corvallis, OR.

U.S. EPA (U.S. Environmental Protection Agency) (1978) Bioassay Procedures for the Ocean Disposal Permit Program, EPA-600/9-78-010, Environmental Research Laboratory, Gulf Breeze, FL.

U.S. EPA (U.S. Environmental Protection Agency) (1982) Pesticide Assessment Guidelines, Subdivision J Hazard Evaluation: Non-target plants, EPA 540/9-82-020, Washington, DC.

U.S. EPA (U.S. Environmental Protection Agency) (1985) Toxic substances control act test guidelines; Final rules. Federal Register 50: 39252 – 39516 (Part 796 – Chemical fate testing guidelines, Part 797 – Environmental effects testing guidelines).

U.S. EPA (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, EPA 540/9-85-134.

U.S. EPA (Environmental Protection Agency) (1996) OPPTS Series 850 Ecological Effects Test Guidelines, Public Draft. At: http://www.epa.gov/opptsfrs/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/index.html

U.S. EPA (U.S. Environmental Protection Agency) (2002) Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, Fourth Edition, Office of Water, Washington, DC, EPA-821-R-02-013, 335 pp.

U.S. EPA (U.S. Environmental Protection Agency) (2003) Ambient Aquatic Life Water Quality Criteria for Atrazine – Revised Draft, October, 2003, EPA-R-03-023.

VanEwijk, P.H. and Hoekstra, J.A. (1993) Calculation of the IC50 and its confidence interval when subtoxic stimulus is present, *Ecotoxicology and Environmental Safety* **25**:25-32.

Walsh, G.E. and Alexander, S.V. (1980) A marine algal bioassay method: results with pesticides and industrial wastes, *Water, Air and Soil Pollution* **13**:45-55.

Abbreviations

AAP	Algal Assay Procedure
a.i.	active ingredients
ASTM	American Society for Testing and Materials, also known as ASTM International.
CAS number	Chemical Abstracts Service (Registry) number
EDTA	ethylenediamine tetraacetate
LOEC	lowest observed effect concentration
OECD	Organization for Economic Co-operation and Development
PAR	photosynthetically active radiation
NOEC	no observed effect concentration
ppt	parts per thousand
U.S. EPA	United States Environmental Protection Agency.