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Toxic Substances

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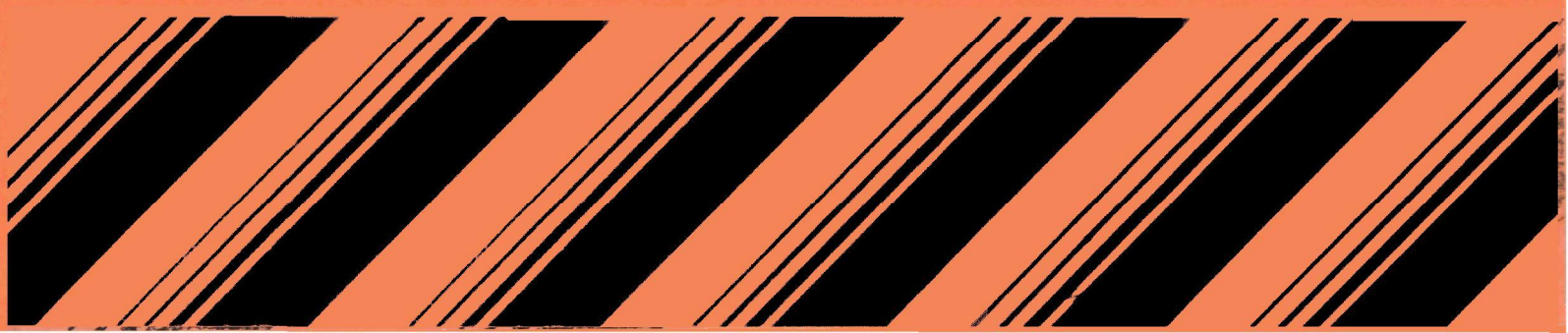
Environmental Effects Test Guidelines

Part One



GUIDELINES AND SUPPORT DOCUMENTS

FOR

ENVIRONMENTAL EFFECTS TESTING

Part One

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

PREAMBLE

The following guidelines describe methods for performing testing of chemical substances under the Toxic Substances Control Act (TSCA). These methods include the state-of-the-art for evaluating certain properties, processes and effects of chemical substances. They are intended to provide guidance to test sponsors in developing test protocols for compliance with test rules issued under Section 4 of the TSCA. They may also provide guidance for testing which is unrelated to regulatory requirements. Support documentation is included for some of these guidelines. It is expected that additional guidelines and support documentation will be incorporated later as the state-of-the-art evolves or the need for them warrants.

Since these guidelines are divided into three sections which cover the diverse areas of health effects, environmental effects and chemical fate testing, there are some differences in the ways they are presented. These differences are explained in an introduction prepared for each section.

INTRODUCTION TO ENVIRONMENTAL EFFECTS TESTING GUIDELINES

In order to assess the environmental risk associated with a chemical substance, a determination of ecotoxicity potential, along with information on the transport and fate of the substance in the environment, is needed. The extent and degree to which a chemical may pose a potential hazard can be characterized in part by its ecotoxicity to plant, animal and microbial species which are valued for economic or ecological importance. Ecotoxicity can be evaluated on the basis of those acute, subacute or chronic effects which result in death, inhibition of reproduction, or an impairment of growth and development of an organism. Ecotoxicity also can occur as a result of the presence or accumulation of a chemical in or on one organism which is not affected by the chemical but serves as basic food source for another organism which is affected.

Whether a chemical substance will cause ecotoxic effects is greatly dependent upon the organism and the stage in the life cycle in which exposure occurs and the conditions under which exposure occurs. The toxicity of a chemical may not be the same to all organisms or to all levels of biological organization. Ideally, such testing should employ test organisms or systems which provide for the broadest range of taxonomic representation and biological processes within the constraints of the costs and resources available. The Test Guidelines for Environmental Effects Testing have been selected with these constraints in mind.

The Guidelines use single species of plants and animals and

incorporate the best state-of-the-art methodologies for testing purposes. It is anticipated that the species and methodologies used in the Guidelines will be reviewed and revised as the state-of-the-art changes. Such changes may include other single species tests, multi-species tests or microcosm tests.

The Test Guidelines and Support Documents are identified by the prefixes EG and ES, respectively, and are numbered sequentially. Where applicable, each Test Guideline is supported by a document which provides the scientific background and rationale used in the development of the Test Guideline. In some cases, a Support Document provides support for two Test Guidelines.

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EG-1
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DAPHNID ACUTE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
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DAPHNID ACUTE TOXICITY TEST

(a) Purpose. This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub.L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 et seq.). This guideline prescribes an acute toxicity test in which daphnids (Daphnia magna or D. pulex) are exposed to a chemical in static and flow-through systems. The United States Environmental Protection Agency will use data from this test in assessing the hazard a chemical may present in the aquatic environment.

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

(1) "Brood stock" means the animals which are cultured to produce test organisms through reproduction.

(2) "EC50" means that experimentally derived concentration of test substance in dilution water that is calculated to affect 50 percent of a test population during continuous exposure over a specified period of time. In this guideline, the effect measured is immobilization

measured is immobilization.

(3) "Ephippium" means a resting egg which develops under the carapace in response to stress conditions in daphnids.

(4) "Flow-through" means a continuous or an intermittent passage of test solution or dilution water through a test chamber or culture tank with no recycling.

(5) "Immobilization" means the lack of movement by the test organisms except for minor activity of the appendages.

(6) "Loading" means the ratio of daphnid biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber at a point in time, or passing through the test chamber during a specific interval.

(7) "Static system" means a test system in which the test solution and test organisms are placed in the test chamber and kept there for the duration of the test without renewal of the test solution.

(c) Test procedures--(1) Summary of the test. (i) Test chambers are filled with appropriate volumes of dilution water. In the flow-through test, the flow of dilution water through each chamber is adjusted to the rate desired. The test chemical is introduced into each treatment chamber. The addition of test chemical in the flow-through system is conducted at a rate which is sufficient to establish and maintain the desired

concentration in the test chamber. The test is started within 30 minutes afterthe test chemical has been added and uniformly distributed in static test chambers or after the concentration of test chemical in each flow-through test chamber reaches the prescribed level and remains stable. At the initiation of the test, daphnids which have been cultured and acclimated in accordance with the test design are randomly placed into the test chambers. Daphnids in the test chambers are observed periodically duringthe test, the immobile daphnids removed, and the findings recorded.

(ii) Dissolved oxygen concentration, pH, temperature, the concentration of test chemical and other water quality parameters are measured at specified intervals in selected test chambers. Data are collected during the test to develop concentration-response curves and determine EC50 values for the test chemical.

(2) [Reserved]

(3) Range-finding test. (i) A range-finding test should be conducted to establish test solution concentrations for the definitive test.

(ii) The daphnids should be exposed to a series of widelyspaced concentrations of the test chemical (e.g., 1, 10, 100 mg/l, etc), usually under static conditions.

(iii) A minimum of five daphnids should be exposed to each

concentration of test chemical for a period of 48 hours. The exposure period may be shortened if data suitable for the purpose of the range-finding test can be obtained in less time. No replicates are required and nominal concentrations of the chemical are acceptable.

(4) Definitive test. (i) The purpose of the definitive test is to determine the concentration-response curves and the 24- and 48- hour EC50 values with the minimum amount of testing beyond the range-finding test.

(ii) A minimum of 20 daphnids per concentration should be exposed to five or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32 and 64 mg/l). An equal number of daphnids should be placed in two or more replicates. If solvents, solubilizing agents or emulsifiers have to be used, they should be commonly used carriers and should not possess a synergistic or antagonistic effect on the toxicity of the test chemical. The concentration of solvent should not exceed 0.1 ml/l. The concentration ranges should be selected to determine the concentration-response curves and EC50 values at 24 and 48 hours. Concentration of test chemical in test solutions should be analyzed prior to use.

(iii) Every test should include controls consisting of the same dilution water, conditions, procedures and daphnids from the

same population (culture container), except that none of the chemical is added.

(iv) The dissolved oxygen concentration, temperature and pH should be measured at the beginning of the test and at 24 and 48 hours in each chamber.

(v) The test duration is 48 hours. The test is unacceptable if more than 10 percent of the control organisms appear to be immobilized, stressed or diseased during the 48 hour test period. Each test chamber should be checked for immobilized daphnids at 3, 6, 12, 24 and 48 hours after the beginning of the test. Concentration-response curves and 24-hour and 48-hour EC50 values for immobilization should be determined along with their 95 percent confidence limits.

(vi) In addition to immobility, any abnormal behavior or appearance should also be reported.

(vii) Distribution of daphnids among test chambers should be randomized. In addition, test chambers within the testing area should be positioned in a random manner or in a way in which appropriate statistical analyses can be used to determine the variation due to placement.

(viii) The concentration of dissolved test chemical (that which passes through a 0.45 micron filter) in the chambers should be measured as often as is feasible during the test. In the

static test the concentration of test chemical should be measured, at a minimum, at the beginning of the test and at the end of the test in each test chamber. In the flow-through test the concentration of test chemical should be measured at a minimum; (A) in each chamber at the beginning of the test and at 24 and 48 hours after the start of the test; (B) in at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system. Among replicate test chambers of a treatment concentration, the measured concentration of the test chemical should not vary more than 20 percent (+ or -).

(5) [Reserved]

(6) Analytical measurements--(i) Test chemical. Deionized water should be used in making stock solutions of the test chemical. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test chemical in a sample should be validated before beginning the test by appropriate laboratory practices. An analytical method is not acceptable if likely degradation products of the test chemical, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) Numerical. The number of immobilized daphnids should

be counted during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves. A 24- and 48- hour EC50 and corresponding 95 percent interval should be calculated.

(d) Test conditions--(1) Test species--(i) Selection. (A) The cladocerans, Daphnia magna or D. pulex, are the test species to be used in this test. Either species may be used for testing of a particular chemical. The species identity of the test organisms should be verified using appropriate systematic keys. First instar daphnids, $\leq$ 24 hours old, are to be used to start the test.

(B) Daphnids to be used in acute toxicity tests should be cultured at the test facility. Records should be kept regarding the source of the initial stock and culturing techniques. All organisms used for a particular test should have originated from the same source and be from the same population (culture container).

(C) Daphnids should not be used for a test (1) if cultures contain ephippia; (2) if adults in the cultures do not produce young before day 12; (3) if more than 20 percent of the culture stock die during the two days preceeding the test; (4) if adults in the culture do not produce an average of at least three young per adult per day over the seven day period prior to the test

and (5) if daphnids have been used in any portion of a previous test, either in a treatment or in a control.

(ii) Acclimation. (A) Daphnids should be maintained in 100 percent dilution water at the test temperature for at least 48 hours prior to the start of the test. This is easily accomplished by culturing them in the dilution water at the test temperature. Daphnids should be fed prior to the test.

(B) During culturing and acclimation to the dilution water, daphnids should be maintained in facilities with background colors and light intensities similar to those of the testing area.

(iii) Care and handling. (A) Daphnids should be cultured in dilution water under similar environmental conditions to those used in the test. Organisms should be handled as little as possible. When handling is necessary it should be done as gently, carefully and quickly as possible. During culturing and acclimation, daphnids should be observed carefully for ephippia and other signs of stress, physical damage and mortality. Dead and abnormal individuals should be discarded. Organisms that touch dry surfaces or are dropped or injured in handling should be discarded.

(B) Smooth glass tubes (I.D. greater than 5 mm) equipped with rubber bulb should be used for transferring daphnids

with minimal culture media carry-over. Care should be exercised to introduce the daphnids below the surface of any solution to avoid trapping air under the carapace.

(iv) Feeding. A variety of foods (e.g., unicellular green algae) have been demonstrated to be adequate for daphnid culture. Daphnids should not be fed during testing.

(2) Facilities--(i) Apparatus. (A) Facilities needed to perform this test include: (1) containers for culturing and acclimating daphnids; (2) a mechanism for controlling and maintaining the water temperature during the culturing, acclimation, and test periods; (3) apparatus for straining particulate matter, removing gas bubbles, or aerating the water as necessary; and (4) an apparatus for providing a 16-hour light and 8-hour dark photoperiod with a 15 - 30 minute transition period. In addition, the flow-through system should contain appropriate test chambers in which to expose daphnids to the test chemical and an appropriate test substance delivery system.

(B) Facilities should be well ventilated and free of fumes and disturbances that may affect the test organisms.

(C) Test chambers should be loosely covered to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions.

(ii) Construction materials. (A) Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from the dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect the test results.

(B) For static tests, daphnids can be conveniently exposed to the test chemical in 250 ml beakers or other suitable containers.

(C) For flow-through tests, daphnids can be exposed in glass or stainless steel containers with stainless steel or nylon screen bottoms. The containers should be suspended in the test chamber in such a manner to insure that the test solution flows regularly into and out of the container and that the daphnids are always submerged in at least five centimeters of test solution. Test chambers can be constructed using 250 ml beakers or other suitable containers equipped with screened overflow holes, standpipes or V-shaped notches.

(iii) Dilution water. (A) Surface or ground water, reconstituted water or dechlorinated tap water are acceptable as dilution water if daphnids will survive in it for the duration of the culturing, acclimation and testing periods without showing signs of stress. The quality of the dilution water should be constant and should meet the following specifications:

SUBSTANCE	MAXIMUM CONCENTRATION
Particulate matter	20 mg/liter
Total organic carbon or	2 mg/liter
chemical oxygen demand	5 mg/liter
Un-ionized ammonia	1 ug/liter
Residual chlorine	<3 ug/liter
Total organophosphorus pesticides	50 ng/liter
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs) or	50 ng/liter
organic chlorine	25 ng/liter

(B) The above water quality parameters under paragraph (d)(2)(iii)(A) of this section should be measured at least twice a year or whenever it is suspected that these characteristics may have changed significantly. If dechlorinated tap water is used, daily chlorine analysis should be performed.

(C) If the diluent water is from a ground or surface water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) should be measured. Reconstituted water can be made by adding specific amounts of reagent-grade chemicals to deionized or distilled water. Glass distilled or carbon-filtered deionized water with a conductivity less than 1 u ohm/cm is acceptable as the diluent for making reconstituted water.

(iv) Cleaning. All test equipment and test chambers should be cleaned before each test using standard laboratory procedures.

(v) Test substance delivery system. In flow-through tests, proportional diluters, metering pump systems or other suitable devices should be used to deliver test chemical to the test chambers. The system should be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test chemical in each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow through a test chamber should be equal to at least five times the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent from any one test chamber to another or from one time to any other.

(3) Test parameters. Environmental parameters of the water contained in test chambers should be maintained as specified below:

(i) Temperature of 20 ± 1°C.

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation. Aeration, if needed to achieve this level, should be done before the addition of the test chemical. All treatment and control chambers should be given the same aeration treatment.

(iii) The number of daphnids placed in a test chamber should not affect test results. Loading should not exceed forty daphnids per liter test solution in the static system. In the flow-through test, loading limits will vary depending on the flow rate of dilution water. Loading should not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 16 hours light and 8 hours darkness, with a 15-30 minute transition period.

(e) Reporting. The sponsor should submit to the USEPA all data developed by the test that are suggestive or predictive of acute toxicity and all concomitant gross toxicological manifestations. In addition to the reporting requirements prescribed in Part 792--Good Laboratory Practice Standards, the reporting of test data should include the following:

(1) The name of the test, sponsor, testing laboratory, study director, principal investigator and dates of testing.

(2) A detailed description of the test chemical including its source, lot number, composition (identity and con-centration or major ingredients and major impurities), known physical and chemical properties and any carriers or other additives used and their concentrations.

(3) The source of the dilution water, its chemical

characteristics (e.g., conductivity, hardness, pH, etc.) and a description of any pretreatment.

(4) Detailed information about the daphnids used as brood stock, including the scientific name and method of verification, age, source, treatments, feeding history, acclimation procedures and culture method. The age (in hours) of the daphnids used in the test is also reported.

(5) A description of the test chambers, the volume of solution in the chambers, the way the test was begun (e.g., conditioning, test chemical additions), the number of test organisms per test chamber, the number of replicates per treatment, the lighting, the method of test chemical introduction or the test substance delivery system and the flow rate (in flow-through test) expressed as volume additions per 24 hours.

(6) The concentration of the test chemical in each test chamber at times designated for static and flow-through tests.

(7) The number and percentage of organisms that were immobilized or showed any adverse effects in each test chamber at each observation period.

(8) Utilizing the average measured test chemical concentration, concentration-response curves should be fitted to immobilization data at 24 and 48 hours. A statistical test of goodness-of-fit should be performed and the results reported.

(9) The 24- and 48- hour EC50 values and their respective 95 percent confidence limits using the mean measured test chemical concentration and the methods used to calculate both the EC50 values and their confidence limits.

(10) All chemical analyses of water quality and test chemical concentrations, including methods, method validations and reagent blanks.

(11) The data records of the culture, acclimation and test temperatures.

(12) Any deviation from this test guideline and anything unusual about the test, e.g., diluter failure, temperature fluctuations, etc..

EG-2
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DAPHNID CHRONIC TOXICITY TEST

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WASHINGTON, D.C. 20460

DAPHNID CHRONIC TOXICITY TEST

(a) Purpose. This guideline is intended for use in developing data on the chronic toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (P.L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 et seq.). This guideline prescribes a chronic toxicity test in which daphnids are exposed to a chemical in a renewal or a flow-through system. The United States Environmental Protection Agency will use data from this test in assessing the hazard a chemical may present to the aquatic environment.

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

(1) "Brood stock" means the animals which are cultured to produce test organisms through reproduction.

(2) "Chronic toxicity test" means a method used to determine the concentration of a substance in water that produces an adverse effect on a test organisms over an extended period of time. In this test guideline, mortality and reproduction (and optionally, growth) are the criteria of toxicity.

(3) "EC50" means that experimentally derived concentration of test substance in dilution water that is calculated to affect 50 percent of a test population during continuous exposure over a specified period of time. In this guideline, the effect measured is immobilization.

(4) "Ephippium" means a resting egg which develops under the carapace in response to stress conditions in daphnids.

(5) "Flow-through" means a continuous or intermittent passage of test solution or dilution water through a test chamber or culture tank with no recycling.

(6) "Immobilization" means the lack of movement by daphnids except for minor activity of the appendages.

(7) "Loading" means the ratio of daphnid biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber at a point in time or passing through the test chamber during a specific interval.

(8) "MATC (Maximum Acceptable Toxicant Concentration)" means the maximum concentration at which a chemical can be present and not be toxic to the test organism.

(9) "Renewal system" means the technique in which test organisms are periodically transferred to fresh test solution of the same composition.

(c) Test procedure--(1) Summary of the test. (i) Test

chambers are filled with appropriate volumes of dilution water. In the flow-through test the flow of dilution water through each chamber is then adjusted to the rate desired. The test substance is introduced into each test chamber. The addition of test substance in the flow-through system is done at a rate which is sufficient to establish and maintain the desired concentration of test substance in the test chamber.

(ii) The test is started within 30 minutes after the test substance has been added and uniformly distributed in the test chambers in the renewal test or after the concentration of test substance in each test chamber of the flow-through test system reaches the prescribed level and remains stable. At the initiation of the test, daphnids which have been cultured or acclimated in accordance with the test design, are randomly placed into the test chambers. Daphnids in the test chambers are observed periodically during the test, immobile adults and offspring produced are counted and removed, and the findings are recorded. Dissolved oxygen concentration, pH, temperature, the concentration of test substance, and other water quality parameters are measured at specified intervals in selected test chambers. Data are collected during the test to determine any significant differences ($P \leq 0.05$) in immobilization and reproduction as compared to the control.

(2) [Reserved]

(3) Range-finding test. (i) A range-finding test should be conducted to establish test solution concentrations for the definitive test.

(ii) The daphnids should be exposed to a series of widely spaced concentrations of the test substance (e.g., 1, 10, 100 mg/l), usually under static conditions.

(iii) A minimum of five daphnids should be exposed to each concentration of test substance for a period of time which allows estimation of appropriate chronic test concentrations. No replicates are required and nominal concentrations of the chemical are acceptable.

(4) Definitive test. (i) The purpose of the definitive test is to determine concentration-response curves, EC50 values and effects of a chemical on immobilization and reproduction during chronic exposure.

(ii) A minumum of 20 daphnids per concentration should be exposed to five or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, 64 mg/l). An equal number of daphnids should be placed in two or more replicates. The concentration ranges should be selected to determine the concentration-response curves, EC50 values and MATC. Solutions should be analyzed for chemical concentration

prior to use and at designated times during the test.

(iii) Every test should include controls consisting of the same dilution water, conditions, procedures and daphnids from the same population (culture container), except that none of the chemical is added.

(iv) The test duration is 21 days. The test is unacceptable if:

(A) more than 20 percent of the control organisms appear to be immobilized, stressed or diseased during the test;

(B) each control daphnid living the full 21 days produces an average of less than 60 young;

(C) any ephippia are produced by control animals.

(v) The number of immobilized daphnids in each chamber should be recorded on days 7, 14, 21 of the test. After offspring are produced, they should be removed from the test chambers every two or three days. Counts of the cumulative number of offspring per adult (number of young divided by the number of adults in each chamber) and the cumulative number of immobilized offspring per adult should be recorded on days 14, and 21 of the test. Concentration-response curves, EC50 values and associated 95 percent confidence limits for adult immobilization should be determined for days 7, 14 and 21. A MATC should be determined for the most sensitive test criteria

measured (number of adult animals immobilized, number of young per adult and number of immobilized young per adult).

(vi) In addition to immobility, any abnormal behavior or appearance should also be reported.

(vii) Distribution of daphnids among the test chambers should be randomized. In addition, test chambers within the testing area should be 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) [Reserved]

(6) Analytical measurements--(i) Test chemical. Deionized water should be used in making stock solutions of the test substance. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test substance in a sample should be validated before beginning the test by appropriate laboratory practices. An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) Numerical. The number of immobilized adults, total offsring per adult and immobilized offspring per adult should be

counted during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for the adult immobilization concentration-response curves calculated on days 7, 14 and 21. A 7-, 14- and 21-day EC50, based on adult immobilization and corresponding 95 percent confidence intervals, should be calculated. Appropriate statistical tests (e.g., analysis of variance, mean separation test) should be used to test for significant chemical effects on chronic test criteria (cumulative number of immobilized adults, cumulative number of offspring per adult and cumulative number of immobilized offspring per adult) on days 7, 14 and 21. An MATC should be calculated using these chronic test criteria.

(d) Test conditions--(1) Test species. (i) Selection. (A) The cladocerans, Daphnia magna or D. pulex, are the species to be used in this test. Either species can be utilized for testing of a particular chemical. The species identity of the test organisms should be verified using appropriate systematic keys.

(B) First instar daphnids, $\leq$ 24 hours old, are to be used to start the test.

(ii) Acquisition. (A) Daphnids to be used in chronic toxicity tests should be cultured at the test facility. Records should be kept regarding the source of the initial stock and

culturing techniques. All organisms used for a particular test should have originated from the same population (culture container).

(B) Daphnids should not be used for a test if:

(1) cultures contain ephippia;

(2) adults in the cultures do not produce young before day 12;

(3) more than 20 percent of the culture stock die in the two days preceding the test;

(4) adults in the culture do not produce an average of at least three young per adult per day over the seven day period prior to the test;

(5) daphnids have been used in any portion of a previous test either in a treatment or in a control.

(iii) Feeding. (A) During the test the daphnids should be fed the same diet and with the same frequency as that used for culturing and acclimation. All treatments and control(s) should receive, as near as reasonably possible, the same ration of food on a per-animal basis.

(B) The food concentration depends on the type used. Food concentrations should be sufficient to support normal growth and development and to allow for asexual (parthenogenic) reproduction. For automatic feeding devices, a suggested rate is

5-7 mg food (either solids or algal cells, dry weight) per liter dilution water or test solution. For manual once-a-day feeding, a suggested rate is 15 mg food (dry weight) per liter dilution water or test solution.

(iv) Loading. The number of test organisms placed in a test chamber should not affect test results. Loading should not exceed forty daphnids per liter in the renewal system. In the flow-through test, loading limits will vary depending on the flow rate of the dilution water. Loading should not cause the dissolved oxygen concentration to fall below the recommended level.

(v) Care and handling of test organisms. (A) Daphnids should be cultured in dilution water under similar environmental conditions to those used in the test. A variety of foods have been demonstrated to be adequate for daphnid culture. They include algae, yeasts and a variety of mixtures.

(B) Organisms should be handled as little as possible. When handling is necessary it should be done as gently, carefully and quickly as possible. During culturing and acclimation, daphnids should be observed carefully for ephippia and other signs of stress, physical damage and mortality. Dead and abnormal individuals should be discarded. Organisms that touch dry surfaces or are dropped or injured during handling should be

discarded.

(C) Smooth glass tubes (I.D. greater than 5mm) equipped with a rubber bulb can be used for transferring daphnids with minimal culture media carry-over.

(D) Care should be exercised to introduce the daphnids below the surface of any solution so as not to trap air under the carapace.

(vi) Acclimation. (A) Daphnids should be maintained in 100 percent dilution water at the test temperature for at least 48 hours prior to the start of the test. This is easily accomplished by culturing them in the dilution water at the test temperature. Daphnids should be fed the same food during the test as is used for culturing and acclimation.

(B) During culturing and acclimation to the dilution water, daphnids should be maintained in facilities with background colors and light intensities similar to those of the testing area.

(2) Facilities--(i) General. (A) Facilities needed to perform this test include:

(1) containers for culturing and acclimating daphnids;

(2) a mechanism for controlling and maintaining the water temperature during the culturing, acclimation and test periods;

(3) apparatus for straining particulate matter, removing gas

bubbles, or aerating the water when water supplies contain particulate matter, gas bubbles, or insufficient dissolved oxygen, respectively;

(4) an apparatus for providing a 16-hour light and 8-hour dark photoperiod with a 15- to 30-minute transition period;

(5) an apparatus to introduce food if continuous or intermittent feeding is used;

(6) in addition, the flow-through test should contain appropriate test chambers in which to expose daphnids to the test substance and an appropriate test substance delivery system.

(B) Facilities should be well ventilated and free of fumes and other disturbances that may affect the test organisms.

(ii) Test chambers. (A) Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from the dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect test results.

(B) For renewal tests, daphnids can be conveniently exposed to the test solution in 250 ml beakers or other suitable containers.

(C) For flow-through tests daphnids can be exposed in glass or stainless steel containers with stainless steel or nylon screen bottoms. Such containers should be suspended in the test

chamber in such a manner to ensure that the test solution flows regularly into and out of the container and that the daphnids are always submerged in at least 5 centimeters of test solution. Test chambers can be constructed using 250 ml beakers or other suitable containers equipped with screened overflow holes, standpipes or V-shaped notches.

(D) Test chambers should be loosely covered to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions.

(iii) Test substance delivery system. (A) In the flow-through test, proportional diluters, metering pump systems or other suitable systems should be used to deliver the test substance to the test chambers.

(B) The test substance delivery system used should be calibrated before and after each test. Calibration includes determining the flow rate through each chamber and the concentration of the test substance in each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow rate through a test chamber should be equal to at least five times the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent from any one test chamber to another or from one

time to any other. For the renewal test, test substance dilution water should be completely replaced at least once every three days.

(iv) Dilution water. (A) Surface or ground water, reconstituted water, or dechlorinated tap water are acceptable as dilution water if daphnids will survive in it for the duration of the culturing, acclimation, and testing periods without showing signs of stress. The quality of the dilution water should be constant and should meet the following specifications:

Substance	Maximum Concentration
Particulate matter	20 mg/l
Total organic carbon or	2 mg/l
chemical oxygen demand	5 mg/l
Un-ionized ammonia	20 ug/l
Residual chlorine	<3 ug/l
Total organophosphorus pesticides	50 ng/l
Total organochlorine pesticides	
plus polychlorinated biphenyls (PCBs)	50 ng/l
or organic chlorine	25 ng/l

(B) The water quality characteristics listed above should be measured at least twice a year or when it is suspected that these characteristics may have changed significantly. If dechlorinated tap water is used, daily chlorine analysis should be performed.

(C) If the diluent water is from a ground or surface water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) should be measured. Reconstituted water can be made by adding specific amounts of reagent-grade chemicals to deionized or distilled water. Glass distilled or carbon filtered deionized water with a conductivity of less than 1 microohm/cm is acceptable as the diluent for making reconstituted water.

(D) If the test substance is not soluble in water an appropriate carrier should be used.

(v) Cleaning of test system. All test equipment and test chambers should be cleaned before each test following standard laboratory procedures. Cleaning of test chambers may be necessary during the testing period.

(3) Test parameters. (i) Environmental conditions of the water contained in test chambers should be maintained as specified below:

(A) Temperature of 20 ± 1°C.

(B) Dissoved oxygen concentration between 60 and 105 percent saturation. Aeration, if needed to achieve this level, should be

done before the addition of the test substance. All treatment and control chambers should be given the same aeration treatment.

(C) Photoperiod of 16-hours light and 8-hours darkness, with a 15-30 minute transition period.

(ii) Additional measurements include:

(A) The concentration of dissolved test substance (that which passes through a 0.45 micron filter) in the chambers should be measured during the test.

(B) At a minimum, the concentration of test substance should be measured as follows:

(1) in each chamber before the test;

(2) in each chamber on days 7, 14 and 21 of the test;

(3) in at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system. Among replicate test chambers of a treatment concentration, the measured concentration of the test substance should not vary more than 20 percent.

(C) The dissolved oxygen concentration, temperature and pH should be measured at the beginning of the test and on days 7, 14 and 21 in each chamber.

(e) Reporting. The sponsor should submit to the USEPA all data developed by the test that are suggestive or predictive of chronic toxicity and all associated toxicologic manifestations.

In addition to the reporting requirements prescribed in the Part 792--Good Laboratory Practice Standards the reporting of test data should include the following:

(1) The name of the test, sponsor, testing laboratory, study director, principal investigator, and dates of testing.

(2) A detailed description of the test substance including its source, lot number, composition (identity and concentration of major ingredients and major impurities), known physical and chemical properties, and any carriers or other additives used and their concentrations.

(3) The source of the dilution water, its chemical characteristics (e.g., conductivity, hardness, pH), and a description of any pretreatment.

(4) Detailed information about the daphnids used as brood stock, including the scientific name and method of verification, age, source, treatments, feeding history, acclimation procedures, and culture methods. The age (in hours) of the daphnids used in the test should be reported.

(5) A description of the test chambers, the volume of solution in the chambers, the way the test was begun (e.g. conditioning, test substance additions), the number of test organisms per test chamber, the number of replicates per

treatment, the lighting, the renewal process and schedule for the renewal chronic test, the test substance delivery system and flow rate expressed as volume additions per 24 hours for the flow-through chronic test, and the method of feeding (manual or continuous) and type of food.

(6) The concentration of the test substance in test chambers at times designated for renewal and flow-through tests.

(7) The number and percentage of organisms that show any adverse effect in each test chamber at each observation period.

(8) The cumulative adult and offspring immobilization values and the progeny produced at designated observation times, the time (days) to first brood and the number of offspring per adult in the control replicates and in each treatment replicate.

(9) All chemical analyses of water quality and test substance concentrations, including methods, method validations and reagent blanks.

(10) The data records of the culture, acclimation, and test temperatures.

(11) Any deviation from this test guideline, and anything unusual about the test, (e.g., dilution failure, temperature fluctuations).

(12) The MATC to be reported is calculated as the geometric mean between the lowest measured test substance concentration

that had a significant ($P<0.05$) effect and the highest measured test substance concentration that had no significant ($P>0.05$) effect on day 7, 14 or 21 of the test. The most sensitive of the test criteria (number of adult animals immobilized, the number of young per female and the number of immobilized young per female) is used to calculate the MATC. The criterion selected for MATC computation is the one which exhibits an effect (a statistically significant difference between treatment and control groups; $P<0.05$) at the lowest test substance concentration for the shortest period of exposure. Appropriate statistical tests (analysis of variance, mean separation test) should be used to test for significant test substance effects. The statistical tests employed and the results of these tests should be reported.

(13) Concentration-response curves utilizing the average measured test substance concentration should be fitted to cumulative adult immobilization data at 7, 14, and 21 days. A statistical test of goodness-of-fit should be performed and the results reported.

(14) An EC50 value based on adult immobilization with corresponding 95 percent confidence limits when sufficient data are present for days 7, 14, and 21. These calculations should be made using the average measured concentration of the test substance.

ES-1
August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

DAPHNID ACUTE AND CHRONIC TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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Technical Support Document for Daphnid Acute and Chronic Tests

I. Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-1 which uses Daphnia species to evaluate the acute and chronic toxicity of chemical substances. The Document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures and conditions prescribed in the Test Guideline. Technical issues and practical considerations relevant to the Test Guideline are discussed. In addition, estimates of the cost of conducting the test are provided.

II. Scientific Aspects

A. Test Procedures

1. General. Relatively few industrial chemicals, compared to the vast number produced, have been previously tested by standard aquatic bioassay methods. As a result, many cannot be classified as to their toxicological properties. The acute and chronic toxicity tests will provide some of the information needed to evaluate the hazard posed to aquatic organisms from a chemical substance. Although assessment of effects on higher levels of biological organization is desirable, it is necessary to begin with effects on individuals and small test populations. Acute effects can be considered as those which cause rapid damage to the organism by the fastest acting mechanism of poisoning which can prove detrimental unless the animal escapes the toxic environment at an early time.

The static acute toxicity test provides the most easily

obtainable measure of toxicity. This test provides information on the immediate effects of short term exposure to potential toxicants under carefully controlled conditions. A single exposure to a potential toxicant in a confined system may represent the worst possible test case for weak or non-motile organisms which may be incapable of avoiding the chemical under natural conditions (Curtis et al. 1979).

The acute flow-through toxicity test is especially applicable for those test substances which display high oxygen demand, are highly volatile, are unstable in aqueous solution, are biodegradable or are readily assimilated by the test organism. By constantly maintaining test solution concentrations and environmental factors such as dissolved oxygen and pH within narrow limits, a more representative indication of the potential toxic effects of the test substance is obtained as opposed to static test conditions where degradation products and metabolic wastes may affect the test organism as well as the test substance.

The proposed Daphnia chronic toxicity test guidelines are designed to assess the effects of test substances on the survival and reproduction of Daphnia as a representative macroinvertebrate. The duration of the test permits the organism to be exposed to a chemical from shortly after birth until well into adulthood. The organisms are exposed long enough to allow the adults to produce several broods of second generation progeny. Initiating exposure shortly after birth allows an assessment of the possible effects of the test substance on such metabolic processes as reproductive system, maturation, fecundity, and growth.

Exposure of the test organisms for more than a complete generation cycle (approximately ten days at the test temperature, Beisinger et al. 1974) allows the testing facility to assess and predict the potential effect of the test compound on a representative Daphnia population. The number of first generation test organisms unaffected at the termination of the test provide an indication of survivorship. Mathematical treatment of the fecundity and survival data provides an index of the inherent ability of a population of test organisms to increase under similar environmental conditions (Boughey,1973). Although the chronic assays require more time and expense, they provide much more accurate predictive information concerning the effects of the test substance (Liptak 1974). The potential disadvantages of the static assays, which exclude feeding of test organisms, include physico-chemical modifications of the test substance and stress on the organisms from their own metabolic wastes, dissolved oxygen depletion, and starvation. These disadvantages are greatly reduced by renewing the test solution-dilution water medium at various rates while providing sufficient food.

The replacement of the medium can be continuous or at fairly frequent intervals. Flow-through tests are more expensive because they require more dilution water, more test substance, and more expensive apparatus. The setup, breakdown, and maintenance time for flow-through systems is greater and requires more experienced personnel.

Intermediate results can be obtained between flow-through and static bioassays by renewing the medium at periodic intervals. This system may be satisfactory if

water is limited, if labor costs are not too high and if bioassays are conducted relatively infrequently. Laboratories that perform bioassays on a regular basis may find flow-through tests to be cheaper and more effective than renewal tests (Liptak 1974).

The difference in results between renewal and flow-through chronic assays may be significant, depending in part on the renewal frequency and the physical characteristics of the test substance. Nebeker and Puglisi (1974) investigated the effects of several PCB's on Daphnia magna using weekly renewal and flow-through techniques. The LC50 values for Arochlor 1254 for three-week assays were 31 ppb in the renewal test and 1.3 ppb in the flow-through test. The authors attribute the thirty-fold difference in toxicity to cumulative toxicity, the volatility of the PCB's and sorption to bacteria, algal waste materials, test container and food surfaces.

The frequency of test chamber renewal may also affect test results. Bunner and Halcrow (1977) observed a significant difference in ephippia production and mortality with different dilution water replacement frequencies. Daphnia magna maintained under the same photoperiod, feeding rate, temperature, and density, but with renewal on an alternate day basis, exhibited 1.2 percent ephippial production and 3.8 percent mortality. Daphnids maintained under a weekly replacement scheme exhibited 8.7 percent ephippial production and 35 percent mortality at the end of the test period. The renewal system test guideline recommends alternate day renewal in order to maintain the best practicable conditions for the test organisms.

The choice of an acute test duration of 48-hours was chosen for the acute tests because of certain biological characteristics of daphnids. Adema (1978) compared the mortality of one-day old daphnids which were fed an algal diet to those which were not fed, under identical conditions. Adema demonstrated that 48 hours is the maximum time which instars can be deprived of food without suffering increased mortality. The 48 hour test duration also allows sufficient time for the instars to molt at least once. Lee and Buikema (1979) report there is a molt-enhanced sensitivity to certain compounds.

The events in the daphnid life cycle are dependent on a number of factors including temperature, food concentration and type. Four distinct periods may be recognized in the daphnid life cycle (Pennak 1978): egg, juvenile, adolescent, and adult. There are few juvenile instars. Generally, D. pulex has 3-4 instars, and D. magna 3-5. The adolescent period is a single instar prior to the first adult instar during which the eggs reach full development in the ovary. The number of adult instars varies. D. pulex generally has 18 - 25 instars, while D. magna from 6 - 22. Molting occurs at an approximate rate of once a day for the juveniles and at a rate of every two days for the adults under favorable conditions.

Richman (1958) observed that D. pulex released the first brood at age eight days at 20°C. Anderson and Jenkins (1942) noted that D. magna released its first brood at the age of seven days at 25°C. Anderson (1932) recorded sexual maturity in D. magna from six to ten days when raised in a temperature range of 18 to 23°C.

The test duration of 21 days, starting with first instar daphnids, will expose the daphnids to the test substance for approximately 11 total instars, including approximately seven adult instars (Anderson and Jenkins 1942, Richman 1958). The total number of instars in the treatments will be a function of the effects of the test substance on juvenile growth and survival, reproductive maturation, and adult growth and survival.

Anderson et al. (1937), using D. pulex, and Anderson and Jenkins (1942) using D. magna, observed a peak in the number of living young produced in both species at the 10th to 11th instar which corresponds to days 20 to 23 after experimental initiation. Instars subsequent to the 10th and 11th instar generally produced less living young per molt. Richman (1958) using D. pulex did not observe a decrease in the number of young produced until approximately days 24 and 28. The three studies indicate that the test period will provide time for several adult instars and should encompass or closely approach the life stages of maximal reproduction. Winner and Farrell (1976) assessed the reproductive sensitivity of D. pulex to copper and concluded that the experiment could have terminated anytime after the third brood (approximately day 13) without altering conclusions as to the effect of copper on reproduction.

It is important that treatment and controls be monitored closely, especially after approximately day 5 to determine the release time of the first brood, frequency of subsequent broods and the number of living young produced per brood.

1. Range-Finding Test

The concentration range for the definitive tests is normally chosen based on the results of a range-finding test. Range-finding tests with daphnids are usually short-term bioassays which use fewer organisms per test substance concentration than required for the definitive test. In all cases, the range-finding test is conducted to reduce the expense involved without having to repeat a definitive test due to inappropriate test substance concentrations.

2. Definitive test

The results of the definitive test will be used to establish any statistically significant (PL 0.05) differences between the treatments and the control(s) pertaining to survivorship and reproductive capabilities. These parameters should provide an indication of the potential effects of the test substance on representative populations of the test organism.

Certain test parameters as promulgated in the Test Guidelines are more or less inflexible or have a narrow range of acceptable values. Slight variations of these parameters have been demonstrated to have a significant effect on the test organisms. Small variations of temperature, for example, can produce changes in the metabolic rate of daphnids (Kaestner 1970, Bunting 1974), as well as on their response to toxicants (Bunting and Robertson, 1975). Other test parameters establish maximum-minimum criteria or a broad range of acceptable values.

ASTM (1980) concluded that, at present, all conditions do not warrant very precise control without invalidating test results. However, some experimental conditions should

be more narrowly controlled. Therefore, recommendations for test procedures are made which, if followed, will yield results that are scientifically sound without causing unnecessary testing costs.

B. Test conditions

1. Test species

a. Selection. Daphnids are of ubiquitous occurrence and are an important link in many aquatic food chains (Kring and O'Brien 1976, Gulati 1978, Makarewicz and Likens 1979). Species of the genus Daphnia are major components of the freshwater zooplankton throughout the world (Hebert 1978). Because of their predominantly herbivorous nature, the daphnids represent an intermediate trophic linkage between the primary producers and the carnivores and predators of higher trophic levels (Gulati 1978).

Daphnia are found in a variety of aquatic environments, except for rapid streams, brooks and grossly polluted water (Pennak 1978). Two of the most common species found in ponds, lakes and permanent pools are D. magna and D. pulex. D. magna, the largest of the daphnids, is generally found in the northern and western parts of North America while D. pulex is distributed over the entire North American continent (Pennak 1978, Buikema et al. 1976). Both D. magna and D. pulex have been used in toxicity tests. D. magna has been used more extensively due to its large size, ease of culture, short generation time, and sensitivity to toxic compounds (Dewey and Parker 1964, Frear and Boyd 1967, Builkema et al. 1976). Buikema, et al. (1976) maintain that the cosmopolitan distribution of D. pulex, as well as its

adaptability to a wider range of habitats, warrants its use as a test organism.

Daphnia have been used to assess the toxicological properties of a number of different types of compounds for both survival and the possible effects on reproduction: insecticides (Adema 1978); herbicides (Schober and Lampert 1977); organic compounds (Adema 1978; Canton et al 1975); metals (Winner and Farrell 1976; Bertram and Hart 1979, Biesinger and Christensen 1972); PCB's (Nebeker and Puglisi 1974); nitrilotriacetate (NTA) (Biesinger et al. 1974); polyethyleneimine (PEI) (Stroganov et al. 1977).

Daphnids have been shown to be very sensitive organisms for assessing the possible deleterious effects of chemicals on other aquatic forms. D. magna was more susceptible to several xenobiotics tested than other invertebrates and fish tested (Canton eet al. 1975, Leeuwangh 1978). Kenaga (1978) used 75 insecticides and herbicides to assess the comparative toxicology of several different species including birds, rats, fish, shrimp, honeybees, and daphnids as indicators in toxicity screening tests. Comparisons between the test organisms and test substances indicated that the daphnids were extremely sensitive to a number of compounds. Comparisons of the test species, within a chemical class, indicated that the invertebrates (daphnids and shrimp) were the most sensitive test form for the entire spectrum of chemicals tested (Kenaga 1978).

In a subsequent review of the toxicity of more than 30,000 test compounds, Kenaga and Moolenar (1979) demonstrated the enhanced sensitivity of D. magna compared to four species of fish, five species of aquatic vascular

plants, and the alga Chlorella.

The sensitivity of D. magna was compared with that of D. pulex in two sets of experiments (Winner and Farrell 1976, Canton and Adema 1978). The conclusions indicated that the two species do not significantly differ in their sensitivities to the compounds tested.

Winner and Farrell (1976) studied the acute and chronic toxicity of copper (as copper sulfate, pentahydrate) to four species of daphnids. The daphnids used were divided into two groups, the larger species, D. magna and D. pulex, and the smaller species, D. parvula and D. ambigua. It was observed that the acute toxicity of D. magna and D. pulex for copper did not differ significantly (72-hour LC_{50}: D. magna, 86.5 ug/l; D. pulex, 86.0 ug/l; $P > 0.05$). However, two smaller species did display a slightly enhanced sensitivity (72-hour LC_{50}: D. parvula, 72.0 ug/l; D. ambigua, 67.7 ug/l. Comparison of chronic exposure data demonstrated consistent similarities in sensitivity with regard to survival. Survivorship curves for the two lowest test conditions (20 ug/l and 40 ug/l) were never significantly different ($P > 0.05$) from those of the controls for the four species of Daphnia. Enhanced mortality was observed at the next highest concentration (60 ug/l) for all test species. The authors attribute this increase in toxicity between 40 ug/l and 60 ug/l to a saturation of the complexing capacity of the natural pond water used as dilution water.

Canton and Adema (1978) investigated the sensitivity of D. magna, D. pulex and D. cucullata to 15 different compounds (13 organic, 2 inorganic). Comparison of acute

toxicity test data pooled from two different laboratories using the three test species indicated that with only two exceptions (aniline and pentachlorophenol), there were no significant differences ($P > 0.05$) between the species. Because of its size (manageability), as well as its comparable sensitivity, D. magna was designated the daphnid of choice for Dutch laboratory studies.

As data are generated using both species under carefully defined conditins, the intercomparability of the two test species for chronic testing can be assessed in future work.

b. Sources. Daphnids as a group display taxonomically troublesome variations in details of setation and in carapace, head and postabdomen morphology.

Test species may be obtained from field collections, supply houses or established cultures and should be identified and documented. Verification of either species used should be performed using the systematic keys of Brooks (1959) or Pennak (1978) for D. magna, and Brandlova et al. (1974) for D. pulex.

2. Maintenance of Test Species

a. Handling and Acclimation. All organisms used in a test should be of the same species and from the same source to reduce variability of the test results. Laboratory culture of daphnids from a single innoculum provides test organisms of similar history. Reproduction can be restricted to the parthenogenetic production of only females when suitable culture conditions are maintained. This insures a supply of experimental animals with genetic variability limited to the heterozygosity of the parent

(APHA 1975, Kaestner 1970, Pennak 1978). Under proper conditions, such as sufficient food, favorable temperature and uncrowded conditions, D. magna cultures have been maintained for many years (Kaestner 1970). Daphnia from cultures in which ephippia (resting eggs) are being produced should not be used for testing, as the production of ephippia indicates unfavorable culture conditions and production of males.

Several culture methods have been described, with no one method universally accepted. Needham et al. (1959) describes several historical methods of culture, some of which are still utilized. Hutchinson (1967) reviews several cultural schemes using primarily algae as food.

The following references are not meant to be exhaustive, but to provide the testing facilities with some recent information of various culture methods: D'Agastino and Provasoli (1970), algae; Murphy (1970), algae; Burns (1969), mixed algae; Kring and O'Brien (1976), algae; Berge (1978), algae; Canton et al. (1975), algae; Lee and Buikema (1979), mixed algae; Winner and Farrell (1976), algae and vitamins; Schultz and Kennedy (1976), algae and yeast; Dewey and Parker (1964), algae and yeast; Buikema et al. (1976), trout chow pellets; Beisinger and Christensen (1972), trout fry food granules and grass; Fear and Boyd (1967), manure-soil; and Whitten et al. (1976), hard-boiled egg yolk.

It is left to the experience and discretion of the testing facilities to decide which method(s) prove to be the most reliable.

Acclimation to new environmental condition(s) is accomplished by various biochemical and biophysical processes. Capacitive adaptations are those which permit

relative constancy of biological activity over a normal range of environmental parameters. To accommodate the necessary biochemical/physiological changes required for the test organism to adapt to new environmental conditions, the rate of change of the factors should be such as to avoid additional stress.

Once the desired conditions are established, the animals should be held at these conditions for a period of time to determine that no delayed symptoms of stress appear which could bias test results.

The recommended rates of temperature acclimation (1°C/day), plus culturing in dilution water for approximately 21 days, are designed to allow the animals to make the necessary physiological adjustments prior to exposure to the treatments.

Food type, concentration, and feeding rate should approximate test conditions as closely as possible. This allows the animals to adjust to test conditions and yields to the investigator a preliminary assessment of the effectiveness of the feeding regime to be used during the extended test period.

A culture should not be used as a source of test organism if (a) the individuals appear stressed or diseased; (b) it possesses adults that do not produce young by day 12, which would indicate delayed maturation or infertility; (c) it has adults that do not produce an average of at least three young per adult per day over a seven-day period, which would indicate reproductive impairment due to genetic or culture conditions such as crowding, inadequate diet or some pathogen; (d) ephippia are being produced, which would indicate stressful conditions; or (e) mortality exceeds 3

percent during the 48 hours immediately preceding the test.

These criteria are designed to prevent bias in the test results caused by the use of reproductively inferior daphnids, which could result from heredity or a stressed parent. Stressed culture conditions causing metabolic dysfunctions in the parent may be reflected in the test organisms. Such dysfunctions as reduced yolk synthesis could result in an inferior test daphnid.

First instar D. magna or D. pulex are the initial test stage. Animals 0-24 hours old are to be used. This age class can be collected by an overnight separation of gravid females, and insures that all the test organisms will be first-instar, pre-molt.

Dewy and Parker (1964) described a separation chamber consisting of funnels with screen openings. The instars passed through the screen and were collected in receiving jars while the adults remained in the funnel. This method resulted in the production of animals of known age with a minimum of labor and time.

Static acute assays indicate an enhanced sensitivity of first instar daphnids as compared to the later juvenile or early adult stages (Sanders and Cope 1966).

Schultz and Kennedy (1976) and Lee and Buikema (1979), again using static acute assays, demonstrated enhanced sensitivity of Daphnia spp. at molting. Such mechanisms as changes in permeability of the body surface and the incorporation of large volumes of water were postulated to explain the enhanced toxic effect during the molt period.

Exposure of the daphnids shortly after brood release insures exposure to the test substance prior to the first molt. Exposure of the test organism at an early age is

designed to assess any possible effects on the organism which may not manifest themselves until later in the life cycle.

Subjecting the first instar to the test substance allows an evaluation of effects on such physiological processes as ovary maturation (reflected in fertility), fecundity, and the time from brood release to the first post-release molt. The effects on the parent generation can also be reflected in growth rate and survivorship.

b. Feeding. Daphnids should not be fed during the acute tests. The presence of food in the test medium may have several effects: (1) The test substance may be absorbed onto the food particles and either increase or decrease its toxic effects (Adema, 1978). (2) It may alter the dissolved oxygen content by increasing BOD (biological oxygen demand), increase dissolved oxygen by photosynthetic activity, or reduce dissolved oxygen by respiratory demands. (3) Feeding may alter the physiology of the instars and change the uptake and metabolism of the test substance: (4) It may introduce more variability into the test.

Feeding is required during culture of daphnids and in renewal and flow-through chronic tests. Food concentration and type is extremely important because it can affect: (1) the concentration of test substance needed to elicit a response, (2) diurnal dissolved oxygen levels, and (3) the physiological state of the test organism.

A large number of variables concerning feeding are evident from the literature. These include food type(s), feeding rates, use of supplements, frequency of renewal of

test medium and dilution water and any food included in natural dilution waters. Recommended feeding regimes for daphnid testing may be based on the literature compilation in Table 1. Final feeding conditions are left to the discretion of the individual laboratory based on experience and the satisfaction of the test guidelines pertaining to control mortality and minimum number of control progeny. These two criteria are designed to insure, in part, proper feeding techniques during test conditions.

The food used should be sufficient to maintain the test organism in a nutritional state which will support normal metabolic activity and reproductive capabilities. This is advisable in order to avoid introducing starvation as a variable into test results.

Adema (1978) states that the feeding of 4.0×10^7 to 6.0×10^7 Chlorella pyrenoidosa cells per liter per adult D. magna per day is the optimum amount of food for reproduction and was the same concentration used in cultures.

Overfeeding may compromise test results through (1) excessive oxygen demands of the food used; (2) preferential sorption of the test compound as some critical food concentration is reached; (3) filtering rate of the daphnids is reduced or goes to zero. Kring and O'Brien (1976) observed a reduction in the filtering ratefor D. pulex at concentrations in excess of 2.5×10^7 Ankistrodesmus fallatus cells per liter. McMahon (1965) observed a leveling of the filtering rate for D. magna at 1.5×10^8 yeast cells per liter and 1.2×10^8 Chlorella vulgaris cells per liter. Less than optimum feeding can be reflected in reduced production of progeny. Richman (1958), using D.

Table 1: Several feeding regimes used in chronic toxicity using *Daphnia*.

Food Type	Calculated Feeding Rate (amount/l/daphnid/day)	Reference
Chlorella pyrenoidosa	2.5 - 6.0 x 10^7 cells	Adema (1978)
Chlorella pyrenoidosa Yeast	1.3 x 10^7 cells 6.5 x 10^9 cells	Bertram and Hart (1979)
Chlorella pyrenoidosa	4 x 10^7 cells	Berge (1978)
Yeast Extract		
Grass and Trout Pellets	7 mg solids	Biesinger and Christensen (1972)
Yeast	2 mg Yeast	Bunner and Halcrow (1977)
Yeast *Scenedesmus obliquus*	1.2 x 10^5 cells 4 x 10^5 cells	Dewey and Parker (1964)
Yeast and *Scenedesmus acutus*	4 mg	Schober and Lampert (1977)
Chlaymdomonas reinhardti	7.5 mg	Winner and Farrell (1976)

pulex and *Chlamydomonas* spp., observed a three-fold change in the cumulative number of young produced with *Chlamydomonas* concentrations ranging from 25 x 10^3 cells per ml (30 young) to 100 x 10^3 cells per ml (92 young).

Bunner and Halcrow (1977), using *D. magna* fed on yeast

and maintained at the same photoperiod and density, observed an increase in ephippia production of 18.6 percent on a starvation diet (0.025 mg yeast per animal per day) as opposed to 9.6 percent ephippia on a diet of 0.05 mg yeast per animal per day.

3. Facilities

a. Construction Materials. Construction materials and any equipment that may contact stock solutions, test solutions or any water into which the test organisms will be placed should not contain any substances that can be leached into the aqueous medium. Such substances could introduce an error into the test results or stress the test organisms by direct or indirect toxic effects.

Materials and equipment should be chosen to minimize or eliminate the occurrance of sorption and leaching, which may reduce the effective concentration of the test substances and introduce a potential error in test results, or which may introduce contaminants into the system.

b. Test Substance Delivery System. In flow-through tests, the delivery of constant concentrations of test substances is required to reduce variability in test results. Large fluctuations in test substance concentration will give abnormally high or low responses, depending upon the mechanism of toxic actions. Proportional diluters with metering pumps or continuous-flow infusion pumps have been used extensively to maintain constant test substance concentration. For the flow-through acute and life-cycle test guideline, all tests should be conducted in intermittent flows from a diluter or in continuous flow with

the test substance added by an infusion pump. The procedures of Mount and Brungs (1967) and Hansen et al. (1974) are recommended if the test substance can be added without a carrier; the device described by Hansen et al. (1974), if a carrier is necessary; or procedure of Bahner et al. (1975), if pumps are required for continuous flow.

Proportional diluters operate on a sequential filling and emptying of water chambers. The water chambers are calibrated to contain a measured amount of water. Separate water chambers can be provided for test substance and diluent water. Diluent and test substance waters are mixed and delivered to the test aquaria. The cyclic action of the diluent is regulated by a solenoid valve connected to the inflow dilution water. The system is subject to electrical power failure, so an alternate emergency power source is recommended.

The proportional diluter is probably the best system for routine use; it is accurate over extended periods of time, is nearly trouble free, and has fail-safe provisions (Lemke et al. 1978). A small chamber to promote mixing of test substance-containing water and dilution water may be used between the diluter and the test aquaria for each concentration. If replicate chambers are used in this test, separate delivery tubes should be run from this mixing chamber to the appropriate replicate chambers. If an infusion pump is used, a glass baffle should be employed to insure mixing of the test substance and dilution water. Calibration of the test substance delivery system should be checked carefully before and during each test. This should include determining the flow rate through each test aquarium and measuring the concentration of test substance in each

test aquarium. The general operation of the system should be checked twice daily.

The use of municipal water supplies is not recommended. Municipal waters often contain high concentrations of potentially harmful components such as chlorine, chloramines, copper, fluoride, lead, zinc and iron. A carbon filtered dechlorinated water may be acceptable if Daphnia can be cultured in it. Caution should be exercised since municipal water may vary considerably in quality and chemical characteristics associated with seasonal changes (e.g., extensive chlorination following heavy storm activity), different sources and modifications or repairs to the distribution system.

c. Cleaning of Test System. Standard laboratory practices (e.g., USEPA 1974) are recommended to remove dust, dirt, other debris, and residues from test facilities. At the end of a test, test systems should be washed in preparation for the next test. This will prevent chemical residues and organic matter from becoming embedded or absorbed into the equipment. It is also recommended tht any silicon cement which has been exposed to a test substance is replaced prior to future tests to avoid contamination due to sorption properties.

Rinsing and priming the system with dilution water before use (conditioning) allows equilibrium to be reached between the chemicals in the water and the materials of the test system. The test system may sorb or react with substances in the dilution water. Allowing this equilibrium to take place before use lessens the chances of water chemistry changes during a test.

d. Dilution Water. An adequate supply of dilution water is required in which the daphnids will survive, grow, and reproduce. This is necessary to insure that the test organisms will not be stressed or adversely affected by the dilution water.

Dilution water quality should be maintained within a certain range to allow for standardization and comparability of test data. Changes outside the acceptable range recommended may cause undue stress to the test organisms, thus biasing test results. Variations in water chemistry from the recommended range may also interfere chemically with the test compound, either enhancing or diminishing the toxicological properties. Criteria have been established for several heavy metals and pesticides which have been known to produce adverse effects on aquatic organisms (ASTM 1980).

The dilution water should be vigorously aerated prior to use for culturing and testing. The recommended saturation value of 90 to 100 percent should provide sufficient oxygen under most conditions for daphnid metabolic demands, as well as any chemical oxygen demand of the test substance.

Test chambers should not be aerated after the test organisms are introduced to prevent entrapment of air bubbles under the daphnids' carapace.

Natural dilution water should be obtained from an uncontaminated well, spring, or surface water source. Wells and springs generally provide water of fairly constant quality. Surface water sources are more likely to be subjected to point or non-point source loadings. Any peculiarities in local ground and surface water due to

geologic conditions that are not listed in the specifications of the test guideline should be investigated in terms of their effects on the test organisms.

Natural dilution water is usually the most cost-efficient type of water, especially for extensive testing and flow-through systems. Reconstituted water may be prepared using ground or surface water which, in itself, will not maintain daphnids. Reconstituted water has the advantage of having well defined chemical characteristics, due to the specific chemical components defined in its manufacture. Reconstituted water is however, less cost-efficient than natural dilution water for large scale renewal or flow-through testing due to the requirement of a distilling apparatus and labor required to measure and mix the necessary chemical components.

e. Loading. The use of 10 instars per 200 ml test solution is recommended for static acute tests. This loading should insure adequate dissolved oxygen for the duration of the test period. Adema (1978) recommended a loading of 10 ml test solution per instar based on oxygen consumption data for the instar at 20°C. Adema suggested this loading would result in a final dissolved oxygen concentration of 80 percent saturation dilution water. The recommended loading provides twice the amount of test solution suggested by Adema and should provide a margin of safety for the dissolved oxygen of both the organism and chemical.

A recommended loading of one daphnid per test chamber for chronic, reproductive studies is designed to meet the dissolved oxygen requirements of the organism and to allow

the observation of critical stages in the life cycle such as initial release of instars and the subsequent brood frequency and size.

Survivorship studies using five parent daphnids per 200 ml test solution may present some problems with maintaining dissolved oxygen levels above 60 percent saturation, depending on the food type and feeding rate. Should dissolved oxygen values be observed below 60 percent saturation, the use of a larger volume of test solution or a different food type and feeding rate is recommended.

For acute and chronic flow-through assays the near constant changing of the test solution should prove sufficient to maintain all environmental conditions within the criteria for a definitive test.

f. Controls. Controls are required for every test to insure that the observed effects are due to the test substance and not to other factors.

In acute toxicity tests, a maximum of 10 percent immobility is permissible in dilution water control daphnids due to inherent biological factors and possible handling-induced stress. Higher immobilization negates the test results and indicates the need to determine the cause of the increased immobilization in the control. Possible sources of increased control immobility include culturing techniques, acclimation procedures, handling techniques, or testing facilities or procedures.

In chronic tests, to insure that the reproductive capabilities of the test population are not impaired, a lower limit of at least 60 young produced per control animal (cumulative) has been established for the test duration.

Criteria proposed in the test guideline for culture conditions are designed to reduce possible stress conditions which could be reflected in reproductive impairment of the organisms. Similar criteria for the experimental procedures are designed to insure that the observed effects are due to the test compound and not to crowding or feeding. Table 2 presents a summary of observed 21 day cumulative production of young daphnids. The range in values can be attributed to such factors as loading, temperature, food type and concentration, dilution water characteristics, and in some cases, the need to extract 21 day data from the results of experiments of longer duration.

Table 2: Cumulative production of young D. magna during a period of 21 days after birth.

Average Cumulative Production of Young Per Daphnid	Test Temperature °C	Reference
67-122	20	Berge (1978)
73	25	Anderson and Jenkins (1942)
38-48	18	Nebecker and Puglisi (1974)
63	20	Schober and Lampert (1977)
30-92	20	Richman (1958)
88	-	Canton et al. (1975)

g. Carriers. Carriers should only be used when they are necessary to solubilize hydrophobic test compounds. Dimethylformamide and triethylene glycol are the carriers of choice due to their low volatility and toxicity, as well as their ability to dissolve many organic compounds (ASTM 1980). Sax (1979) suggests that dimethylformamide not come in contact with halogenated hydrocarbons and inorganic nitrates due to the reactivity of the compounds. Schober and Lampert (1977) observed a significant effect of the use of ethanol as a carrier for Atrazin (chlorinated triazine herbicide) using D. pulex for a 28-day exposure. Although no effect was observed with 0.1 percent ethanol carrier, the use of 0.5 percent carrier with the herbicide produced effects greater than the sum of the individual effects. Significant differences ($P < 0.05$) were observed on such parameters as number of young per animal and mean length.

Comparison of dilution water controls and 0.5 percent ethanol controls indicated that use of the ethanol control resulted in approximately a 40 percent reduction in the number of young per animal over the experimental period. Mean length data also indicated a carrier effect.

The investigation of Schober and Lampert (1977) demonstrates possible errors associated with the use of a carrier and reinforces the recommendation that a carrier be used only when necessary. The investigation also emphasizes the need for a carrier control and the investigation of the effects of two different concentrations of the same carrier on the test organism.

Krugel et al. (1978) describe an apparatus for the continuous dissolution of poorly soluble compounds for bioassays.

h. Randomization. Randomization is required to prevent conscious or unconscious biases from being introduced. These biases can be in environmental conditions such as temperature and lighting, daphnid selection and distribution.

4. Environmental Conditions

a. Dissolved Oxygen. Daphnia respond to partial anoxia by synthesizing hemoglobin (Hoar 1966, Lockwood 1967, Kaestner 1970). This adaptation has significant survival value (increased life span and increased egg production) when compared to those organisms that lack or possess reduced concentrations of hemoglobin. In mature females, considerable hemoglobin enters the eggs, accelerating embryonic development. After egg laying, the level of hemoglobin in the adult's blood is approximately two-thirds of its normal value. The concentration in the blood increases during the time the young are developing in the brood pouch. The cycle is then repeated with the release of the young and production of a new batch of parthenogenic eggs.

Considering its molecular size, incorporation into the eggs and possible replacement rates, hemoglobin synthesis may represent a considerable energy demand on the organism. Such demands can be inferred from the increase rate of feeding at partial anoxic conditions. No chronic toxicity test data could be located comparing Daphnia response to varying concentrations of dissolved oxygen.

Adema (1978) determined the oxygen consumption of 25 egg-bearing adult daphnids at 20°C to be about 850 ug/02/day. The instars released from adults (25) in 24

hours consume an additional 600 ug/02/day. Adema suggests a minimum of 2.5 liters of oxygen-saturated medium for 25 daphnid on an alternate day renewal scheme will satisfy daphnid oxygen demands, as well as any other oxygen sinks such as food remnants, excreta and test compound oxygen demands.

The solubility of oxygen in freshwater at 20°C is approximately 9 mg/l. The test guidelines recommend that dissolved oxygen not fall below 60 percent saturation or approximately 5.5 mg/l at any time during the test. Kring and O'Brien (1976), using D. pulex at 22°C, observed that when the oxygen concentration dropped below 3 mg/l the filtering rate of D. pulex decreased drastically. The same authors cite unpublished data of a critical oxygen concentration of about 3 mg/l for D. magna. The recommended minimum concentration of 60 percent saturation is well above the critical oxygen concentrations observed.

Kring and O'Brien (1976) observed that exposure for less than one hour to oxygen concentrations of 1 mg/l caused a negligible depression in the filtering rate of D. pulex. Longer exposures to dissolved oxygen values less than the 3 mg/l critical concentration resulted in depressed filtering rates (60 percent reduction) for an eight-hour period. After 24 hours of exposure, the filtration rate increased to near normal values. Continued exposure to dissolved oxygen values less than the critical concentration resulted in the ability of the animals to resume and surpass the initial high filtering rates, presumably because the low oxygen concentration stimulated the production of hemoglobin. The daphnids synthesized increasing amounts of hemoglobin

associated with the time they were exposed to low dissolved oxygen values increased. Consideration of the increase in hemoglobin synthesis and the high filtering rates associated with long-term exposure to low dissolved oxygen suggests that hemoglobin synthesis is energetically inefficient. Maintenance of test dissolved oxygen values above 60 percent saturation should prevent any biochemical stress on the test organism associated with hemoglobin synthesis which could decrease the energy available for other metabolic processes.

The presence or absence of dissolved molecular oxygen in the test solution may also affect the form of the metals and ions in the medium. Several general types of redox reactions of ionic species of metals have been demonstrated, depending in part on pH, the presence of organic complexing agents, the presence of other ionic species such as the carbonate ion and the presence of molecular oxygen (Faust and Hunter 1967). Several of the reactions are reversible; thus various equilibria can be established, depending on the chemical composition of the test medium. Maintenance of dissolved oxygen levels in excess of 60 percent saturation should reduce the variability of the ionic shifting for metallic test substances. No data could be located to allow comparison of the toxicity of the different ionic forms of a given metal. Due to the chemical complexity of the test medium, such as food type and concentration, metabolic products and test system-test substance interaction, maintaining dissolved oxygen values at a given minimum should help to reduce variability in test results.

The concentration of dissolved oxygen should be monitored closely in static acute test chambers to insure

that the oxygen level is above the required minimum. During a test, the chambers are not to be aerated under any circumstances. This is specified to prevent air entrapment under the instar carapace as well as not to enhance volatilization of the test substance. If levels cannot be maintained above 60 percent, the static flow-through method should be used.

The potential for oxygen depletion in the renewal test does exist, and depends on loading, food type and concentration, feeding frequency, and renewal frequency. Several renewal studies listed in Table 3 show a range of values for these parameters. Only one study, Winner and Farrell (1976), presents any dissolved oxygen data. These authors state that observed dissolved oxygen values were always in excess of 95 percent saturation.

Table 3. Summary of Renewal Chronic Assay Techniques.

Initial Loading (daphnids/volume)	Replacement Frequency	Food	Feeding Frequency	Reference
1/40 ml	4 Days	C. pyrenoidosa and Yeast	1 day	Bertram and Hart (1979)
1/40 ml	1 Week	Trout Pellets	1 Week	Biesinger and Christensen (1972)
1/100 ml	2 Days	Scenedesmus and Yeast	2 Days	Schober and Lampert (1977)
1/50 ml	2-3 Days	Chlorella	2-3 Days	Stroganov et al. (1977)
1/40 ml	3 Days	Chlamydomonas	1 Day	Winner and Farrell (1976)

The use of photosynthetically active algae can provide significant oxygen to the test medium during the light photoperiod. During the dark photoperiod, algal respiration competes with the other oxygen sinks for dissolved oxygen. Again, depending on a number of variables, significant oxygen depletion is a possibility. Animals maintained on non-photosynthetic food with extended replacement schemes, such as in Biesinger and Christensen (1972), may be exposed to extended periods of low dissolved oxygen. No data on the effects of low dissolved oxygen on reproduction and survivorship of the test organisms could be located.

The suggested scheme of alternate day renewal in chronic testing should aid in maintaining dissolved oxygen levels above the recommended minimum. Dissolved oxygen readings should be taken several times during the first two days of testing to determine that the proper levels are maintained. It is especially important to determine dissolved oxygen values at the end of the dark photoperiod when using algal food supplies.

The inability to maintain sufficient dissolved oxygen values in a renewal system indicates the need for a flow-through test or larger volume test chambers.

The flow-through techniques should not present any significant dissolved oxygen problems due to (1) the constant replacement of the test medium and (2) the volume of the test chamber and loading ratio, which should provide sufficient oxygen according to the data of Adema (1978). This is not to imply that dissolved oxygen need not be monitored.

b. Light. A 16-hour light, 8-hour dark photoperiod with a 15 to 30-minute transition has been suggested to meet biological requirements of daphnids and to increase test standardization (ASTM, 1980). The recommended photoperiod approximates the temperate summer light regime which would support parthenogenic reproduction. The transition period is recommended to simulate natural conditions. A device for maintaining the photoperiod has been described by Drummond and Dawson (1970).

Stross and Hill (1968), using D. pulex at five individuals per 50 ml density at 19°C for 30 days duration, observed decreasing sexual reproduction with changing photoperiod. At 12L:12D, approximately 90 percent sexual reproduction was recorded, while increasing the light period to 14L:10D resulted in 0 percent sexual reproduction. Extrapolation to the recommended photoperiod at 16L:8D should insure parthenogenic reproduction.

Wide spectrum fluorescent bulbs (Color Rendering Index greater than 90) and a light intensity at the surface of the test chambers not exceeding 800 lux (74 ft. candles) is essentially equivalent to the average tabletop conditions. These facilities would allow the use of room lighting for experimental conditions, provided it was controlled to the recommended photoperiod and transition period.

Temperature. The selected test temperature (20 ± 1°C) approximates room temperature, thus minimizing the requirement for extensive temperature controlling devices in culturing, acclimating and testing facilities.

An accurate device controlling room temperature should maintain the daphnids within the proper range of temperatures.

The selected temperature also approximates summer water temperatures for temperate lakes. The test temperature is within the range in which D. magna reproduces parthenogenically. Kaestner (1970) reports asexual reproduction for D. magna in the temperature range 11°C to 27°C. It was also reported that temperatures below 15°C, together with stress conditions, resulted in sexual reproduction. Sexual reproduction was also reported as temperatures approached 30°C.

Variations in test temperatures beyond those suggested could bias test results. Bunting (1974) observed a 50 percent reduction in the growth rate of juvenile D. magna at 15°C as compared to 20°C. This rate reduction manifested itself in increased time periods between molts. At 25°C an increased growth rate was observed as compared to 20°C, but beyond 25°C a reduction in t e growth rate was observed, indicating a potential thermal stress. Bunting and Robertson (1975) observed a significant difference in the acute effects of two herbicides, Aminotrizole and Amitrole, on juvenile D. pulex at two temperatures. It required approximately twice the herbicide concentration at the lower temperature, 15°C, to produce the same effect as at 20°C. The narrow range of specified temperatures for these test guidelines should reduce significant differences in reported test results.

The information presented in Table 4 was derived from

a search of articles concerning control mortality during chronic toxicity tests. In some cases, the 21-day control mortality was interpolated from long-term survivorship curves. Based on the studies the recommendation of a 20 percent maximum control mortality criterion for 21 day chronic tests should provide a reasonable testing requirement.

Table 4: Percent Mortality in Controls for 21-day experiments using daphnids.

Percent Mortality	Test Temperature	Species	Reference
12	20	D. magna	Berge (1978)
8-20	18	D. magna	Nebecker Puglisi (1974)
0	19	D. pulex	Bertram and Hart (1976)
15	20	D. magna	Winner and Farrell (1976)
0	20	D. pulex	Schober and Lampert (1977)
11	20	D. pulex	Winner and Farrell (1976)

C. Reporting

1. Acute Tests. For each set of data, with a minimum of the 24- and 48- hour observations, the EC50 and 95 percent confidence limits should be calculated based on the mean measured concentration of the toxicant. A concentration-response curve for each observation period shall also be constructed. It is strongly suggested that a statistician be consulted before the test is initiated to insure that the specific test procedures used will satisfy the statistical requirements of the methods of data analysis.

Acute toxicity tests usually produce quantal data, that is, counts of the number of organisms in two mutually-exclusive categories - alive/dead; affected/not affected. A variety of methods can be used to calculate an EC50 and 95 percent confidence limits from quantal data containing two or more concentrations at which the percent affected is between zero and one hundred. The most widely used are the probit moving average, and Litchfield-Wilcoxon methods (Finney, 1964 and 1971, Stephan 1977, Litchfield and Wilcoxon 1949). The method of Litchfield and Wilcoxon (1949) produces a slope function which together with EC50 value allows reconstruction of the probit line. The slope of the straight line can be useful for interpolation of the potential effects of concentrations other than those near the EC50. The slope may also provide indications as to the mode of toxicity or any change in the toxic effects over the experimental period. Sprague (1969) discusses the construction and interpretation of concentration-response curves using several methods of data analysis. Thus, both the EC50 values and the dose-response curves are necessary

for evaluating the hazard potential of a given test substance.

2. Chronic Tests. The statistical methods used to evaluate the effects of a test compound on survival and reproduction should be described in full. The choice of methods is left to the testing facilities, but it is suggested that a statistician be consulted prior to the initiation of the test program.

Suggested methods include analysis of variance (ANOVA) and appropriate mean separation tests (Sokal and Rahlf 1969, Steele and Torrie 1960).

III. Economic Aspects.

The Agency awarded a contract to Enviro Control, Inc. to provide us with an estimate of the cost for performing static and flow-through acute toxicity tests and renewal and flow-through chronic toxicity tests. Enviro Control supplied us with two estimates; a protocol estimate and a laboratory survey estimate.

Protocol Estimates

	range	mean
Acute (static and flow-through)	$322-$965	$643
Chronic (renewal and flow-through)	$2021-$6064	$4043

These estimates were prepared by separating the guidelines into individual tasks and estimating the hours used to accomplish each task. Hourly rates were then applied to yield a total direct labor charge. An overhead rate of 115 percent, other direct costs ($50 - acute, $450 - chronic), a general and administrative rate of 10 percent and a fee of 20 percent were then added to the direct labor charge to yield the final estimate.

Laboratory Survey Estimates

	range	mean
Acute (static and flow-through)	$340-$1250	$743
Chronic (renewal and flow-through)	$750-$10,000	$4178

The laboratory survey estimates were compiled from three laboratories for the acute guideline and five laboratories for the chronic guideline.

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EG-3
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MYSID SHRIMP ACUTE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

MYSID SHRIMP ACUTE TOXICITY TEST

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

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

(1) "Death" means the lack of reaction of a test organism to gentle prodding.

(2) "Flow-through" means a continuous or an intermittent passage of test solution or dilution water through a test chamber or a holding or acclimation tank, with no recycling.

(3) "LC50" means that experimentally derived concentration of test substance that is calculated to kill 50 percent of a test population during continuous exposure over a specified period of

time.

(4) "Loading" means the ratio of test organisms biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber.

(5) "Retention chamber" means a structure within a flow-through test chamber which confines the test organisms, facilitating observation of test organisms and eliminating loss of organisms in outflow water.

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

(c) Test procedures--(1) Summary of the test. In preparation for the test, test chambers are filled with appropriate volumes of dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired. The test substance is introduced into each test chamber. In a flow-through test, the rate at which the test substance is added is adjusted to establish and maintain the desired concentration of test substance in each test chamber. The test is started by randomly introducing mysids acclimated in accordance with the test design into the test chambers. Mysids in the test chambers are observed periodically during the test, the dead mysids removed and the findings recorded. Dissolved oxygen concentration, pH, temperature,

salinity, the concentration of test substance, and other water quality characteristics are measured at specified intervals in test chambers. Data collected during the test are used to develop concentration-response curves and LC50 values for the test substance.

(2) [Reserved]

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

(A) which life stage (juvenile or young adult) is to be utilized in the definitive test.

(B) the test solution concentrations for the definitive test.

(ii) The mysids should be exposed to a series of widely spaced concentrations of test substance (e.g., 1, 10, 100 mg/l, etc.), usually under static conditions.

(iii) This test should be conducted with both newly-hatched juvenile (< 24 hours old) and young adult (5-6 days old) mysids. For each age class (juvenile or young adult), a minimum of ten mysids should be exposed to each concentration of test substance for up to 96 hours. The exposure period may be shortened if data suitable for the purpose of the range-finding test can be obtained in less time. The age class which is most sensitive to the test substance in the range-finding test should

be utilized in the definitive test. When no apparent difference in sensitivity of the two life stages is found, juveniles should be utilized in the definitive test. No replicates are required and nominal concentrations of the chemical are acceptable.

(4) Definitive test. (i) The purpose of the definitive test is to determine the concentration-response curves and the 48- and 96- hour LC50 values with the minimum amount of testing beyond the range-finding test.

(ii) The definitive test should be conducted on the mysid life stage (juveniles or young adults) which is most sensitive to the test substance being evaluated.

(iii) A minimum of 20 mysids per concentration should be exposed to five or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 3 and 64 mg/l). An equal number of mysids should be placed in two or more replicates. If solvents, solubilizing agents or emulsifiers have to be used, they should be commonly used carriers and should not possess a synergistic or antagonistic effect on the toxicity of the test substance. The concentration of solvent should not exceed 0.1 ml/l. The concentration ranges should be selected to determine the concentration-response curves and LC50 values at 48 and 96 hours. The concentration of test substance in test solutions should be analyzed prior to use.

(iv) Every test should include controls consisting of the same dilution water, conditions, procedures, and mysids from the same population or culture container, except that none of the chemical is added.

(v) The dissolved oxygen concentration, temperature, salinity, and pH should be measured at the beginning of the test and at 24, 48, 72 and 96 hours in each chamber.

(vi) The test duration is 96 hours. The test is unacceptable if more than 10 percent of the control organisms die or exhibit abnormal behavior during the 96 hour test period. Each test chamber should be checked for dead mysids at 3, 6, 12, 24, 48, 72 and 96 hours after the beginning of the test. Concentration-response curves and 48- and 96- hour LC50 values should be determined along with their 95 percent confidence limits.

(vii) In addition to death, any abnormal behavior or appearance should also be reported.

(viii) Distribution of mysids among test chambers should be randomized. In addition, test chambers within the testing area should be positioned in a random manner or in a way in which appropriate statistical analyses can be used to determine the variation due to placement.

(ix) The concentration of dissolved test substance (that

which passes through a 0.45 micron filter) in the chambers should be measured as often as is feasible during the test. At a minimum, during static tests, the concentration of test substance should be measured in each chamber at the beginning and at the end of the test. During the flow-through test, the concentration of test substance should be measured (A) in each chamber at the beginning of the test and at 48 and 96 hours after the start of the test; (B) in at least one chamber containing the next to the lowest test substance concentration at least once every 24 hours during the test; and (C) in at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system. Among replicate test chambers of a treatment concentration, the measured concentration of the test substance should not vary more than 20 percent.

(5) [Reserved]

(6) Analytical measurements--(i) Test chemical. Deionized water should be used in making stock solutions of the test substance. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test substance in a sample should be validated before beginning the test by appropriate laboratory practices. An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis

and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) Numerical. The number of dead mysids should be counted during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves. A 48- and 96- hour LC50 and corresponding 95 percent interval should be calculated.

(d) Test conditions--(1) Test species--(i) Selection. (A) The mysid shrimp, Mysidopsis bahia, is the organism specified for these tests. Either juvenile (< 24 hours old) or young adult (5-6 days old) mysids are to be used to start the test.

(B) Mysids to be used in acute toxicity tests should originate from laboratory cultures in order to assure that the individuals are of similar age and experiential history. Mysids used for establishing laboratory cultures may be purchased commercially or collected from appropriate natural areas. Because of similarities with other mysid species, taxonomic verification should be obtained from the commercial supplier or through an appropriate systematic key.

(C) Mysids used in a particular test should be of similar age and be of normal size and appearance for their age. Mysids

should not be used for a test if they exhibit abnormal behavior or if they have been used in a previous test, either in a treatment or in a control group.

(ii) Acclimation. (A) Any change in the temperature and chemistry of the dilution water used for holding or culturing the test organisms to those of the test should be gradual. Within a 24-hour period, changes in water temperature should not exceed 1°C, while salinity changes should not exceed 5 Percent.

(B) During acclimation mysids should be maintained in facilities with background colors and light intensities similar to those of the testing areas.

(iii) Care and handling. Methods for the care and handling of mysids such as those described in USEPA (1978) can be used during holding, culturing and testing periods.

(iv) Feeding. Mysids should be fed during test ng. Any food utilized should support survival, growth and reproduction of the mysids. A recommended food is live Artemia spp. (48-hour-old nauplii).

(2) Facilities--(i) Apparatus. (A) Facilities which may be needed to perform this test include: (1) flow-through or recirculating tanks for holding and acclimating mysids; (2) a mechanism for controlling and maintaining the water temperature during the holding, acclimation and test periods; (3) apparatus

for straining particulate matter, removing gas bubbles, or aerating the water, as necessary; and (4) an apparatus for providing a 14-hour light and 10-hour dark photoperiod with a 15 to 30 minute transition period. In addition, for flow-through tests, flow-through chambers and a test substance delivery system are required. Furthermore, it is recommended that mysids be held in retention chambers within test chambers to facilitate observations and eliminate loss of test organisms through outflow water. For static tests, suitable chambers for exposing test mysids to the test substance are required. Facilities should be well ventilated and free of fumes and disturbances that may affect the test organisms.

(B) Test chambers should be loosely covered to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions.

(ii) Cleaning. Test substance delivery systems and test chambers should be cleaned before each test following standard laboratory practices.

(iii) Construction materials. (A) Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from dilution water and should not contain substances that can be leached into aqueous solution in

quantities that can affect test results.

(B) For use in the flow-through test, retention chambers utilized for confinement of test organisms can be constructed with netting material of appropriate mesh size.

(iv) Dilution water. (A) Natural or artificial seawater is acceptable as dilution water if mysids will survive and successfully reproduce in it for the duration of the holding, acclimating and testing periods without showing signs of stress, such as reduced growth and fecundity. Mysids should be cultured and tested in dilution water from the same origin.

(B) Natural seawater should be filtered through a filter with a pore size of < 20 microns prior to use in a test.

(C) Artificial seawater can be prepared by adding commercially available formulations or by adding specific amounts of reagent-grade chemicals to deionized water. Deionized water with a conductivity less than 1 u ohm/cm at 12°C is acceptable for making artificial seawater. When deionized water is prepared from a ground or surface water source, conductivity and total organic carbon (or chemical oxygen demand) should be measured on each batch.

(v) Test substance delivery system. In flow-through tests, proportional diluters, metering pumps or other suitable systems should be used to deliver test substance to the test chambers.

The system used should be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test substance in each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow through a test chamber should be equal to at least five times the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent among test chambers or across time.

(3) Test parameters. Environmental parameters of the water contained in test chambers should be maintained as specified below:

(i) Temperature of 25 ± 2°C.

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation. Aeration, if needed to achieve this level, should be done before the addition of the test substance. All treatment and control chambers should be given the same aeration treatment.

(iii) The number of mysids placed in a test solution should not be so great as to affect results of the test. Thirty mysids per liter is the recommended level of loading for a static test. Loading requirements for the flow-through test will vary depending on the flow rate of dilution water. The loading should

not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 14 hours light and 10 hours darkness, with a 15-30 minute transition period.

(v) Salinity of 20 ± 3 °/oo.

(e) Reporting. The sponsor should submit to the EPA all data developed during the test that are suggestive or predictive of acute toxicity and all concomitant toxicologic manifestations. In addition to the general reporting requirements prescribed in Part 792--Good Laboratory Practice Standards, the reporting of test data should include the following:

(1) The source of the dilution water, its chemical characteristics (e.g., salinity, pH, etc.) and a description of any pretreatment.

(2) Detailed information about the test organisms, including the scientific name and method of verification, age, source, history, abnormal behavior, acclimation procedures and food used.

(3) A description of the test chambers, the depth and volume of solution in the chamber, the way the test was begun (e.g., conditioning, test substance additions, etc.), the number of organisms per treatment, the number of replicates, the loading, the lighting, the test substance delivery system and the flow

rate expressed as volume additions per 24 hours.

(4) The measured concentration of test substance in test chambers at the times designated.

(5) The number and percentage of organisms that died or showed any other adverse effects in the control and in each treatment at each observation period.

(6) Concentration-response curves should be fitted to mortality data collected at 24, 48, 72 and 96 hours. A statistical test of goodness-of-fit should be performed and the results reported.

(7) The 48-hour and 96-hour LC50, and when sufficient data have been generated, the 24-hour and 72-hour LC50's and the corresponding 95 percent confidence limits and the methods used to calculate the values. These calculations should be made using the average measured concentration of the test substance.

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

(9) The data records of the holding, acclimation and test temperature and salinity.

(f) References. U.S. Environmental Protection Agency, 1978. Bioassay Procedures for the Ocean Disposal Permit Program. Environmental Research Laboratory, Office of Research and Development. Gulf Breeze, Fl. EPA-600-9-78-010.

8 pages

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MYSID SHRIMP CHRONIC TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

Mysid shrimp chronic toxicity test

(a) Purpose. This guideline is intended for use in developing data on the chronic toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub.L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 et seq.). This guideline prescribes tests using mysids as test organisms to develop data on the chronic toxicity of chemicals. The United States Environmental Protection Agency (EPA) will use data from these tests in assessing the hazard of a chemical to the aquatic environment.

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

(1) "Chronic toxicity test" means a method used to determine the concentration of a substance that produces an adverse effect from prolonged exposure of an organism to that substance. In this test, mortality, number of young per female and growth are used as measures of chronic toxicity.

(2) "Death" means the lack of reaction of a test organism to

gentle prodding.

(3) "Flow-through" means a continuous or an intermittent passage of test solution or dilution water through a test chamber or a holding or acclimation tank, with no recycling.

(4) "G1 (Generation 1)" means those mysids which are used to begin the test, also referred to as adults; G2 (Generation 2) are the young produced by G1.

(5). "LC50" means that experimentally derived concentration of test substance that is calculated to kill 50 percent of a test population during continuous exposure over a specified period of time.

(6) "Loading" means the ratio of test organism biomass (gram, wet weight) to the volume (liters) of test solution in a test chamber.

(7) "MATC" (Maximum Acceptable Toxicant Concentration) means the maximum concentration at which a chemical can be present and not be toxic to the test organism.

(8) "Retention chamber" means a structure within a flow-through test chamber which confines the test organisms, facilitating observation of test organisms and eliminating washout from test chambers.

(c) Test procedures--(1) Summary of the test. (i) In preparation for the test, the flow of test solution through each

chamber is adjusted to the rate desired. The test substance is introduced into each test chamber. The rate at which the test substance is added is adjusted to establish and maintain the desired concentration of test substance in each test chamber. The test is started by randomly introducing mysids acclimated in accordance with the test design into retention chambers within the test and the control chambers. Mysids in the test and control chambers are observed periodically during the test, the dead mysids removed and the findings reported.

(ii) Dissolved oxygen concentration, pH, temperature, salinity, the concentration of test substance and other water quality characteristics are measured at specified intervals in selected test chambers.

(iii) Data collected during the test are used to develop a MATC (Maximum Acceptable Toxicant Concentration) and quantify effects on specific chronic parameters.

(2) [Reserved]

(3) Range-finding test. (i) A range-finding test should be conducted to establish test solution concentrations for the definitive test.

(ii) The mysids should be exposed to a series of widely spaced concentrations of the test substance (e.g., 1, 10, 100 mg/l), usually under static conditions.

(iii) A minimum of 10 mysids should be exposed to each concentration of test substance for a period of time which allows estimation of appropriate chronic test concentrations. No replicates are required and nominal concentrations of the chemical are acceptable.

(4) Definitive test. (i) The purpose of the definitive test is to determine concentration-response curves, LC50 values, and effects of a chemical on growth and reproduction during chronic exposure.

(ii) A minimum of 40 mysids per concentration should be exposed to four or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32 and 64 mg/l). An equal number of mysids should be placed in two or more replicates. If solvents, solubilizing agents or emulsifiers have to be used, they should be commonly used carriers and should not possess a synergistic or antagonistic effect on the toxicity of the test substance. The concentration of solvent should not exceed 0.1 ml/l. The concentration ranges should be selected to determine the concentration-response curves, LC50 values and MATC. Concentration of test substance in test solutions should be analyzed prior to use.

(iii) Every test should include controls consisting of the same dilution water, conditions, procedures and mysids from the

same population or culture container, except that none of the chemical is added.

(iv) The dissolved oxygen concentration, temperature, salinity and pH should be measured at the beginning of the test and on days 7, 14, 21 and 28 in each chamber.

(v) The test duration is 28 days. The test is unacceptable if more than 20 percent of the control organisms die, appear stressed or are diseased during the test. The number of dead mysids in each chamber should be recorded on days 7, 14, 21 and 28 of the test. At the time when sexual characteristics are discernable in the mysids (approximately 10-12 days in controls; possible delays may occur in mysids exposed to test substances), the number of males and females (identified by ventral brood pouch) in each chamber should be recorded. Body length (as measured by total midline body length, from the anterior tip of the carapace to the posterior margin of the uropod) should be recorded for males and females at the time when sex can be determined simultaneously for all mysids in control and treatment groups. This time cannot be specified because of possible delays in sexual maturation of mysids exposed to test substances. A second observation of male and female body lengths should be conducted on day 28 of the test. To reduce stress on the mysids, body lengths can be recorded by photography through a stereo-

microscope with appropriate scaling information. As offspring are produced by the G1 mysids (approximately 13-16 days in controls), the young should be counted and separated into retention chambers at the same test substance concentration as the chambers where they originated. If available prior to termination of the test, observations on the mortality, number of males and females and male and female body length should be recorded for the G2 mysids. Concentration-response curves, LC50 values and associated 95 percent confidence limits for the number of dead mysids (G1) should be determined for days 7, 14, 21 and 28. An MATC should be determined for the most sensitive test criteria measured (cumulative mortality of adult mysids, number of young per female, and body lengths of adult males and females).

(vi) In addition to death, any abnormal behavior or appearance should also be reported.

(vii) Distribution of mysids among test chambers should be randomized. In addition, test chambers within the testing area should be positioned in a random manner or in a way in which appropriate statistical analyses can be used to determined the variation due to placement.

(viii) The concentration of dissolved test substance (that which passes through a 0.45 micron filter) in the chambers should

be measured as often as is feasible during the test. The concentration of test substance should be measured: (a) in each chamber at the beginning of the test and on days 7, 14, 21 and 28; and (b) in at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system. Among replicate test chambers of a treatment concentration, the measured concentration of the test substance should not vary more than 20 percent.

(5) [Reserved]

(6) Analytical measurements--(i) Test chemical. Deionized water should be used in making stock solutions of the test substance. Standard analytical methods should be employed whenever available in performing the analyses. The analytical method used to measure the amount of test substance in a sample should be validated before beginning the test by appropriate laboratory practies. An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) Numerical. (A) The number of dead mysids, cumulative young per female and body lengths of male and female mysids should be recorded during each definitive test. Appropriate

statistical analyses should provide a goodness-of-fit determination for the day 7, 14, 21 and 28 adult (G1) death concentration-response curves.

(B) A 7-, 14-, 21- and 28- day LC50, based on adult (G1) death, and corresponding 95 percent confidence intervals should be calculated. Appropriate statistical tests (e.g., analysis of variance, mean separation test) should be used to test for significant chemical effects on chronic test criteria (cumulative mortality of adults, cumulative number of young per female and body lengths of adult male and females) on designated days. An MATC should be calculated using these chronic test criteria.

(d) Test conditions--(1) Test species--(i) Selection. (A) The mysid shrimp, Mysidopsis bahia, is the organism specified for these tests. Juvenile mysids, $\leq$ 24 hours old, are to be used to start the test.

(B) Mysids to be used in chronic toxicity tests should originate from laboratory cultures in order to ensure the individuals are of similar age and experiential history. Mysids used for establishing laboratory cultures may be purchased commercially or collected from appropriate natural areas. Because of similarities with other mysid species, taxonomic determinations should be verified by the commercial supplier or by an appropriate individual.

(C) Mysids used in a particular test should be of similar age and be of normal size and appearance for their age.

(D) Mysids should not be used for a test if they exhibit abnormal behavior, or if they have been used in a previous test, either in a treatment or in a control group.

(ii) Acclimation. (A) Any change in the temperature and chemistry of the water used for holding or culturing the test organisms to those of the test should be gradual. Within a 24-hour period, changes in water temperature should not exceed 1°C, while salinity changes should not exceed 5 Percent.

(B) During acclimation mysids should be maintained in facilities with background colors and light intensities similar to those of the testing areas.

(iii) Care and handling. Methods for the care and handling of mysids such as those described in USEPA (1978) can be used during holding, culturing and testing periods.

(iv) Feeding. Mysids should be fed during testing. Any food utilized should support survival, growth and reproduction of the mysids. A recommended food is live Artemia spp. nauplii (approximately 48 hours old).

(2) Facilities--(i) Apparatus. (A) Facilities which may be needed to perform this test include: (1) flow-through or recirculating tanks for holding and acclimating mysids; (2) a

mechanism for controlling and maintaining the water temperature during the holding, acclimation and test periods; (3) apparatus for straining particulate matter, removing gas bubbles, or aerating the water, as necessary; and (4) an apparatus for providing a 14-hour light and 10-hour dark photoperiod with a 15- to 30-minute transition period. In addition, flow-through chambers and a test substance delivery system are required. It is recommended that mysids be held in retention chambers within test chambers to facilitate observations and eliminate loss through outflow water.

(B) Facilities should be well ventilated and free of fumes and disturbances that may affect the test organisms.

(C) Test chambers should be loosely covered to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions.

(ii) Cleaning. Test substance delivery systems and test chambers should be cleaned before each test following standard laboratory practices.

(iii) Construction materials. (A) Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from the dilution water and should not contain substances that can be leached into aqueous solution in

quantities that can affect the test results.

(B) Retention chambers utilized for confinement of test organisms can be constructed with netting material of appropriate mesh size.

(iv) Dilution water. (A) Natural or artificial seawater is acceptable as dilution water if mysids will survive and successfully reproduce in it for the duration of the holding, acclimating and testing periods without showing signs of stress, such as reduced growth and fecundity. Mysids should be cultured and tested in dilution water from the same origin.

(B) Natural seawater should be filtered through a filter with a pore size of < 20 microns prior to use in a test.

(C) Artificial seawater can be prepared by adding commercially available formulations or by adding specific amounts of reagent-grade chemicals to deionized or glass-distilled water. Deionized water with a conductivity less than 1 u ohm/cm at 12°C is acceptable as the diluent for making artificial seawater. When deionized water is prepared from a ground or surface water source, conductivity and total organic carbon (or chemical oxygen demand) should be measured on each batch.

(v) Test substance delivery system. Proportional diluters, metering pumps or other suitable systems should be used to deliver test substance to the test chambers. The system used

should be calibrated before each test. Calibration includes determining the flow rate and the concentration of the test substance in each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow rate through a chamber should be equal to at least five times the volume of the chamber. The flow rates should not vary more than 10 percent among chambers or across time.

(3) Test parameters. Environmental parameters of the water contained in test chambers should be maintained as specified below:

(i) Temperature of 25 $\pm$ 2°C.

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation. Aeration, if needed to achieve this level, should be done before the addition of the test substance. All treatment and control chambers should be given the same aeration treatment.

(iii) The number of mysids placed in a test solution should not be so great as to affect results of the test. Loading requirements for the test will vary depending on the flow rate of dilution water. The loading should not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 14 hours light and 10 hours darkness,

with a 15-30 minute transition period.

(v) Salinity of 20 ± 3 °/oo.

(e) Reporting. The sponsor should submit to the EPA all data developed by the test that are suggestive or predictive of chronic toxicity and all concomitant toxicologic manifestations. In addition to the general reporting requirements prescribed in Part 792--Good Laboratory Practice Standards, the reporting of test data should include the following:

(1) The source of the dilution water, its chemical characteristics (e.g., salinity, pH, etc.) and a description of any pretreatment.

(2) Detailed information about the test organisms, including the scientific name and method of verification, average length, age, source, history, observed diseases, treatments acclimation procedures and food used.

(3) A description of the test chambers, the depth and volume of solution in the chamber, the way the test was begun (e.g., conditioning, test substance additions, etc.), the number of organisms per treatment, the number of replicates, the loading, the lighting, the test substance delivery system, and the flow rate expressed as volume additions per 24 hours.

(4) The measured concentration of test substance in test chambers at the times designated.

(5) The first time (day) that sexual characteristics can be observed in controls and in each test substance concentration.

(6) The length of time for the appearance of the first brood for each concentration.

(7) The means (average of replicates) and respective 95 percent confidence intervals for:

(A) Body length of males and females at the first observation day (depending on time of sexual maturation) and on day 28.

(B) Cumulative number of young produced per female on day 28.

(C) Cumulative number of dead adults on day 7, 14, 21 and 28.

(D) If available prior to test termination (day 28), effects on G2 mysids (number of males and females, body length of males and females and cumulative mortality).

(8) The MATC is calculated as the geometric mean between the lowest measured test substance concentration that had a significant ($P<0.05$) effect and the highest measured test substance concentration that had no significant ($P>0.05$) effect in the chronic test. The most sensitive of the test criteria for

adult (G1) mysids (cumulative number of dead mysids, body lengths of males and females or the number of young per female) is used to calculate the MATC. The criterion selected for MATC computation is the one which exhibits an effect (a statistically significant difference between treatment and control groups; $P<0.05$) at the lowest test substance concentration for the shortest period of exposure. Appropriate statistical tests (analysis of variance, mean separation test) should be used to test for significant chemical effects. The statistical tests employed and the results of these tests should be reported.

(9) Concentration-response curves should be fitted to the cumulative number of adult dead for days 7, 14, 21 and 28. A statistical test of goodness-of-fit should be performed and the results reported.

(10) An LC50 value based on the number of dead adults with corresponding 95 percent confidence intervals for days 7, 14, 21 and 28. These calculations should be made using the average measured concentration of the test substance.

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

(12) The data records of the holding, acclimation and test temperature and salinity.

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

ES-2
August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

MYSID SHRIMP ACUTE AND CHRONIC TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

Table of Contents

I. Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-2 which uses Mysid shrimp to evaluate the toxicity of chemical substances. The Document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures and conditions prescribed in the Test Guideline. Technical issues and practical considerations relevant to the Test Guideline are discussed. In addition, estimates of the cost of conducting the test are provided.

II. Scientific Aspects

A. Test Procedures

1. General

The choice of mysid toxicity tests (96-hour static, 96-hour flow-through or 28-day chronic is based on several considerations. A static test requires less equipment, fewer chemical analyses and disposal of smaller quantities of contaminated wastewater than flow-through systems. Static tests are a relatively easy means to evaluate and compare the acute effects of a test substance.

The flow-through system more closely simulates the natural exposure process, eliminating problems associated with accumulation of organic material and toxic metabolic products. Test substances are more thoroughly mixed in a flow-through system and problems of sorption to suspended sediments, feces and uneaten food are reduced. In order to produce valid toxicity test results, the flow-through test should be used with test substances which have a high oxygen demand, are highly volatile, are unstable, biodegradable or

are removed in significant amounts by the test organisms.

A more comprehensive evaluation of the potential environmental hazard of a test substance is available from chronic toxicity testing. The chronic test using mysids provides two important advantages over other toxicity testing regimes. First, it permits an evaluation of response to chronic exposure to a test substance. Second, it allows a determination of effects of the test substance throughout sequential life stages of the organism (juvenile, adult, egg). These data can be used to estimate potential adverse population and community changes associated with shifts in growth and reproductive potential.

For the acute tests, 96 hours is a convenient interval of time for starting and completing a test in a normal five-day work week, and is better than shorter periods for estimating accumulative and other chronic effects. Because set-up is the most expensive portion of a test, a 96-hour test is only slightly more expensive than 24 or 48 hour tests. Yet additional data on the LC50's over time and the observations of other abnormal effects that do not appear in shorter tests are gained for this slight increase in cost. Although the 48 hour test can reduce costs, eliminate the necessity of feeding of the mysids during the test, and make the test more comparable to the 48 hour Daphnia acute toxicity tests, the 96-hour toxicity test was selected for the mysid test guidelines because of greater potential for determining the incipient LC50 (threshold limit for acute toxicity) through extension of the toxicity curve. In situations where the 96 hour LC50 does not permit estimation of an incipient LC50 (lethal threshold concentration),

continuation of the test may allow better estimation of the toxic effects of the test substance. In a review of 375 toxicity tests, Sprague (1969) found that a lethal threshold clearly had not been reached in 42 tests, while in 122 other tests the threshold was reached in four days or longer. Because of the importance of the incipient LC50 in hazard assessment, continuation of the acute toxicity tests past the 96 hours is recommended for those test substances which do not elicit a threshold concentration within the four day test period.

For the life cycle test, a 28-day experimental period is used to permit testing through at least one complete life cycle in Mysidopsis bahia at 25°C. Juveniles utilized for tests reach sexual maturity within 12-14 days under normal conditions at this temperature (USEPA 1978). However, test substances may delay sexual maturity several days (Nimmo et al. in press c). Tests longer than 28 days are not recommended because of possible fouling of retention chambers with subsequent decreases in the efficiency of the flow-through system.

2. Range-Finding Test

The concentration range for the definitive test is normally chosen based on the results of a range-finding test. In the acute static and flow-through test, the range-finding test also serves as a means of determining which life stage (juvenile or young adult) is most sensitive to the test substance and should be used in the definitive test. For the acute tests, range-finding tests are normally short-term (24-96 hour), static or flow-through bioassays, which utilize fewer organisms per test substance

concentration than the definitive test. For the life cycle test, range-finding tests may take the form of acute, flow-through tests using different life stages or determination of incipient LC50's to allow selection of appropriate definitive test concentrations. In all cases, the range-finding test is conducted to reduce the expense involved with having to repeat a definitive test due to inappropriate test substance concentrations.

3. Definitive Test

The concentration range for the definitive test is chosen based on the results of preliminary range-finding tests. By testing a minimum of five concentrations in the acute bioassays, partial kills both above and below the median 50 percent mortality level are probable and will help define the concentration-response relationship. The more partial kills, the better the definition of the concentration-response curve. The slope and shape of the concentration-response curve may give insight into possible mechanisms of action of a chemical and will allow estimation of the effects of lower concentrations upon test organisms. In addition, by having partial kill data, a greater array of statistical methods can be used to determine the LC50.

The utilization of the most sensitive of the two life stages (juvenile or young adult), as required for the definitive acute mysid tests, is based on evidence that these two life stages exhibited differential mortality to eleven pesticides (Nimmo et al. in press b). The procedure of testing both stages in the range-finding test and using the most sensitive life stage in the definitive test permits

determination of toxicity data at less expense than conducting two complete definitive tests.

Because of the increased expense of the 28-day mysid life cycle test, the nature of the recorded data and equipment limitations, testing of a minimum of only four concentrations is required. These observations will allow determination of the MATC (maximum acceptable toxicant concentration) limits for the most sensitive life cycle criterion recorded (mortality, body lengths of males and females and numbers of young per female).

The number of mysids exposed to each test substance concentration (e.g. 20 in acute tests and 40 in chronic test) is designed to allow adequate numbers for statistical evaluation even with the presence of partial mortality (Nimmo et al. 1977, 1978, USEPA 1978).

Measurement of test substance concentrations at designated periods during static and flow-through tests allows documentation of real test concentrations at appropriate periods under acute and chronic conditions.

Chemical and physical parameters (temperature, pH, dissolved oxygen and salinity) are recorded at specified times to permit evaluation of the biological conditions present for mysid survival in test solutions.

Specified observations on mortality and life cycle characteristics are designed to allow an adequate evaluation of concentration-response effects in both acute and chronic mysid tests. In addition, these defined observation times allow greater comparability of dose-response data between different chemicals and laboratories.

B. Test Conditions

1. Test Species

a. Selection

The primary considerations in the selection of this organism for toxicity testing were: (a) sensitivity to a variety of chemical substances; (b) geographical distribution and abundance, (c) ecological importance and (d) existence of established culture methods for laboratory rearing.

The test species, Mysidopsis bahia, is a member of the family Mysidae, which is found in most of the neritic zones of the world's oceans. Mysidopsis bahia inhabits shallow water grass flats along the eastern and western Gulf of Mexico. They are particularly abundant from the Galveston Bay system to southern Florida.

Mysids occupy an important position in near shore food webs. They constitute a major source of food for many fish species, including catfish, flounder, anchovy, silverside, sunfish and seatrout (Darnell 1958, Schuster 1959, Odum and Herald 1972, Powell and Schwartz 1979). In addition to their role in food chains of fish, mysids are important in the conversion of organic detritus to living tissue in estuarine environments (Hopkins, 1965).

To date, Mysidopsis bahia has been the most extensively tested mysid shrimp (Nimmo et al. 1977, 1978, In press a, in press b, in press c, USEPA 1978, Cripe et al. in press). During its development as a test species (since 1977) methods of culturing, holding and testing have been established, and the methodologies developed at the EPA Gulf Breeze Environmental Research Laboratory were considered

heavily in the design of this guideline. This laboratory, and others, have established Mysidopsis bahia as a test organism and have stressed the importance of the following qualities: (1) small size; (2) short (18 day) life cycle; (3) small brood size; (4) readily reared through all life stages in culture; (5) commercially available (6) representative of an ecologically important family and (7) extremely sensitive to a variety of test substances. In addition, this species has been selected as a test organism for a variety of assessment programs in EPA and other government agencies, as well as private laboratory testing. The sensitivity characteristic of mysids was dramatically reported by Bionomics (EPA Contract No. 68-01-4646). In testing of the acute toxicity (no effects concentrations) of 35 priority pollutants, mysids were found to be on the average more sensitive than any of the other species tested (i.e., Selenestrum capricornutum, Skeletonema costatum, Daphnia magna, Cyprinodon variegatus and Lepomis machrochirus).

b. Sources

It is recommended that the mysids used for laboratory testing as specified in this test guideline be obtained commercially from a supplier willing to certify proper taxonomic identification. Although field collection is acceptable, it is highly recommended that test organisms originate from culture stock. Definitive identification of Mysidopsis bahia (Molenock, 1969), is difficult without expertise, and it occurs sympatrically with two other species of Mysidopsis. Reliable use of Mysidopsis bahia is required for this testing procedure, and it is therefore

suggested that the mysids be cultured in the laboratory to meet all testing needs. The ease with which this species can be cultured in the laboratory has been demonstrated (Nimmo et al. 1977, 1978).

Furthermore, laboratory culturing of mysids permits the isolation of newly-hatched juveniles. This allows control of the size, age and experiential history of mysids used for acute and chronic testing.

2. Maintenance of Test Species

a. Handling and Acclimation

The bay mysid, M. bahia, may be cultured in aquaria supplied with either filtered flowing or recirculating seawater. Details of M. bahia culture can be obtained from Nimmo et al. (1977) and USEPA (1978). A salinity of 20 °/oo for mysid culture allows optimal reproductive conditions (Nimmo et al. 1977, USEPA 1978) and reduces acclimation problems related to transfer of animals from culture to test water.

b. Feeding

Artemia spp. nauplii suggested for mysid feeding, can be reared in the laboratory from commercially available eggs. These eggs or any other appropriate food used for mysids, should not be used if the total organochlorine pesticide plus polychlorinated biphenyls exceeds 0.3 ug/g (wet weight), or if organic chlorine exceeds 0.15 ug/g (wet weight). A recent study by Johns and Walten (1979) reported that adult Mysidopsis bahia fed Artemia spp. from San Pablo Bay, California exhibited increased mortality, did not reproduce and showed reduced growth rates. In contrast, both juvenile and adult mysids fed Artemia spp. strains

collected from Brazil, Australia, Italy and Utah maintained high survival and growth rates. Additional studies have indicated major differences in the nutritional value of Artemia to brachyuran crustaceans (Johns et al. in press). These results strongly imply that nutritional quality of Artemia, possibly associated with pesticide or heavy metal contamination, can significantly influence test results and, therefore, should be considered.

In order to separate Artemia spp. nauplii (used for mysid feeding) from their egg cases and other debris, a light box may be employed. This system makes use of the positive phototropism of Artemia to separate nauplii from unwanted materials. It is important to isolate the nauplii from the egg cases and to deliver only nauplii to the test chambers in order to minimize build-up of organic debris within the chambers. The decomposition of the entrapped egg cases may directly or indirectly enhance or reduce the toxicity of the test substance.

To isolate juvenile mysids, ovigerous females may be placed within a retention chamber, which is then submerged into a five-liter glass battery jar or other suitable vessel (USEPA 1978). The retention chamber should be slightly smaller than the battery jar and should extend above the water level of the battery jar. A slow flow of salt water (approximately 4 drops/second) should be dripped into the jar to sustain proper dissolved oxygen levels and prevent stagnation. Juveniles pass through the cylinder mesh (one millimeter mesh opening) at birth and attach to the walls of the battery jar; thereby minimizing cannibalism by adult females and facilitating capture. During this isolation

procedure, mysids should be given a supply of 24-hr old Artemia spp. (nauplii). Juvenile mysids may be removed from the sides of the jar every 4-12 hours during this period using a glass tube.

3. Facilities

a. General

In flow-through systems it may be necessary to have the capability to vary and maintain the water temperature. Since the temperature of the dilution water can be expected to vary both daily and seasonally, facilities for adjusting temperature may be needed to maintain the desired culturing, holding, and testing temperatures. Filters are needed to remove particulates and biological material from the dilution water so the diluter system and retention chambers will not become clogged, cause a change in test substance concentrations, or lead to stagnation and oxygen depletion within the retention chambers. The primary concern is to minimize the confounding of results associated with the differential sorption of the test substance on cell walls, clay particles, etc. which in turn may enhance or reduce the availability of the test substance to the mysids.

To minimize these problems, the dilution water should be filtered through a 20 micrometer or smaller pore-size filter to sufficiently reduce the amount of suspended sediments, organic material and biological organisms (phytoplankton, zooplankton, fungi, bacteria, etc.).

Requiring filtration through a 20 micrometer or smaller filter is based on recent modifications to the testing procedure developed at the EPA Gulf Breeze Environmental Research Laboratory (USEPA 1978). In addition, filtration

to five micrometers more effectively controls fouling of the retention chamber mesh walls. Minimizing this problem also prevents dissolved oxygen depletion and stagnation within the retention chambers. Filtration through five micrometer filters is attainable for the recommended flow rate and quantity of water needed to conduct the test.

Gas accumulation may also cause adverse effects and therefore, a device which removes air bubbles may be necessary. A suitable device is described by Penrose and Squires (1976). When the dissolved oxygen in the dilution water is less that 60 percent, a device is needed to aerate the water. Culture techniques recommend 70-100 percent saturation for other marine crustaceans (Forster and Beard of the test substance during aeration through volatilization, aeration should be conducted prior to introduction of the test substance.

In order to attain optimal test results, it is necessary to culture and test the organisms in an environment considerate of both their behavioral and physiological needs. Mysid shrimp are extremely sensitive to fluctuations in these parameters which may be reflected in a number of ways, all of which can affect test validity (Bahner et al. 1975).

b. Construction Materials

All pipes, tanks, holding chambers, mixing chambers, metering devices, and test chambers should be made of materials that minimize the release of chemical contaminants into the dilution water or the adsorption of the test substances. Chemicals that may leach from construction materials can stress test organisms, or possibly act

synergistically or antagonistically with test substances to give inaccurate results. Generally, undesirable substances are not leached from borosilicate glass, titanium, and perfluorocarbon plastic. In addition, the tendency of these materials to absorb substances is minimal. Rubber, polyvinyl chloride, copper, brass, galvanized metal, lead and epoxy resins should not come in contact with dilution water, stock solution, or test solutions because of the toxic substances they contain. Cast iron should not be used in water systems since rust may develop and result in fouling. Teflon (Algoflon), Perspex, Polyethylene, Tygon, Polypropylene, Polycarbonates (Makrolor) and Polyester (Gabraster) have been shown to be non-toxic and suitable for experiments with marine organisms (APHA 1975, USEPA 1978).

Retention chambers, aquaria delivery systems, pipes and any tank exposed to solutions that may come in contact with the organism should not be soldered or brazed, since lead, tin, copper or zinc may be leached. Silicone adhesive is the preferred bonding agent for all construction materials. It is inert, and the solvent it generally contains (acetic acid) is easily washed away or volatilized from the system. A minimum amount of the adhesive should contact the test solution because it may absorb test materials. If large amounts of the adhesive are needed for strength, it should be applied to the outsides of chambers and apparatus to minimize contact.

In static testing, borosilicate glass, crystallizing dishes, or similar containers may be used as test chambers. Use of these dishes will minimize sorption of the test substance into the chamber walls and minimize residues of

test substances or metabolites remaining after cleaning. Glass plates can be placed over the test chambers to allow for stacking and to minimize space requirements and evaporative loss of test solution. Chamber size should be adequate to insure proper loading and to minimize cannibalism.

c. Test Substance Delivery System

In flow-through tests, the delivery of constant concentrations of test substances is required to reduce variability in test results. Large fluctuations in test substance concentration will give abnormally high or low responses, depending upon the mechanism of toxic actions. Proportional diluters with metering pumps or continuous flow infusion pumps have been used extensively to maintain constant test substance concentration. For the flow-through acute and life cycle test guideline, all tests should be conducted in intermittent flows from a diluter or in continuous flow with the test substance added by an infusion pump. The procedures of Mount and Brungs (1967) and Hansen et al. (1974) are recommended if the test substance can be added without a carrier; the device described by Hansen et al. (1974) if a carrier is necessary; or procedure of Bahner et al. (1975) if pumps are required for continuous flow.

Proportional diluters operate on a sequential filling and emptying of water chambers. The water chambers are calibrated to contain a measured amount of water. Separate water chambers can be provided for test substance and diluent water. Diluent and test substance waters are mixed and delivered to the test aquaria. The cyclic action of the diluent is regulated by a solenoid valve connected to the

inflow dilution water. The system is subject to electrical power failure, so an alternate emergency power source is recommended. In addition, the mysids should to be shielded from the clicking sound of solenoid valves. If unshielded from this disturbance, mysids may jump out of the test solution and stick to the sides of the retention chambers (USEPA 1978).

The proportional diluter is probably the best system for routine use; it is accurate over extended periods of time, is nearly trouble free, and has fail-safe provisions (Lemke et al. 1978). A small chamber to promote mixing of test substance-containing water and dilution water may be used between the diluter and the test aquaria for each concentration. If replicate chambers are used in this test, separate delivery tubes should be run from this mixing chamber to the appropriate replicate chambers. If an infusion pump is used, a glass baffle should be employed to insure mixing of the test substance and dilution water. Calibration of the test substance delivery system should be checked carefully before and during each test. This should include determining the flow rate through each test aquarium and measuring the concentration of test substance in each test aquarium. The general operation of the system should be checked twice daily.

d. Test Chambers

Retention chambers are suggested to prevent escape of mysids from the test system, reduce cannibalism and facilitate counting and observation. Overcrowding enhances cannibalism and assignment of five mysids per retention chamber is recommended to minimize this problem. The mesh

size (315 micrometer mesh opening) of the screen used in construction of the retention chambers at the EPA laboratory in Gulf Breeze minimizes the problems associated with fouling by fungal, bacterial and algal growth, yet is still small enough to retain the mysids and food organisms. This mesh size is slightly more porous than the 200 micrometer opening mesh recommended by Nimmo et al. (1977). Use of the larger mesh size combined with 20 micrometer filtration obviates the need for continuous lighting, which was employed in early testing protocols (USEPA 1978) to minimize fouling of the retention chambers.

e. Cleaning of Test System

Standard laboratory Practices (e.g, USEPA 1974) are recommended to remove dust, dirt, other debris, and residues from test facilities. At the end of a test, test systems should be washed in preparation for the next test. This will prevent chemical residues and organic matter from becoming embedded or absorbed into the equipment. It is also recommended that any silicon cement which has been exposed to a test substance is replaced prior to future tests to avoid contamination due to sorption properties.

Rinsing and priming the system with dilution water before use (conditioning) allows equilibrium to be reached between the chemicals in the water and the materials of the test system. The test system may sorb or react with substances in the dilution water. Allowing this equilibrium to take place before use lessens the chances of water chemistry changes during a test.

Even after extensive washing, new facilities may still contain toxic residues. The best way to determine whether

toxic residues remain is to rear mysids through at least one complete life cycle. If the mysids survive and successfully reproduce the test facililties can be considered to be free of toxic residues.

f. Dilution Water

A constant supply of dilution water is required to maintain constant experimental conditions. An interruption in flow or change in water quality can change the chemistry of the test system and possibly the response of the test population. Therefore, the results of a test with variable dilution water quality are not comparable to tests run under constant conditions and they are more difficult to interpret.

For acute and chronic toxicity tests, a minimum criterion for acceptable dilution water is that healthy mysids will survive and reproduce in it without showing signs of stress such as abnormal behavior (erratic swimming, loss of equilibrium or lack of feeding activity).

Natural seawater, obtained from a source with similar characteristics as those designated for the test species or water from an area where the test organisms were obtained, is preferable to artificial seawater. Dilution water should be of constant quality and should be uncontaminated. Contaminated water can affect results both directly and indirectly. If natural seawater is used, it should meet the following specifications for contaminant levels (APHA 1975).

Suspended solids	< 20 mg/l
Total organic carbon	< 10 mg/l
Un-ionized ammonia	< 20 mg/l
Residual chlorine	< 3 ug/l
Total Organophosphorous pesticides	< 50 ug/l
Pesticides plus PCB's	< 50 ug/l

In addition, water used to make reconstituted seawater should meet or exceed the same water quality criteria.

Maintenance of desired salinities in the test aquaria throughout the duration of the test may pose a problem. When possible, water from an area of high salinity shoulds be used; low salinities can then be obtained by adding distilled or deionized water as needed. To increase salinity, a strong, natural brine, which can be obtained by freezing and then partially thawing seawater, can be used. This procedure is suitable if limited amounts of seawater are needed; however, it is recommended that artificial seawater salts be used when large increases in salinities are required (APHA 1975).

Due to the volume of water necessary to conduct a chronic test and technical problems associated with conditioning of the dilution water, the use of reconstituted seawater for these tests may not be currently feasible because of high costs and lack of information on proper aging processes. Research is needed to determine methods in which reconstituted water can be conditioned and aged with the use of appropriate storage and selective filtration before use of reconstituted seawater can become a viable alternative.

g. Controls

Controls are required for every test to assure that any effects which are observed are due to the test substance and not to other factors. These may include effects from construction materials, environmental factors, nutritional quality of food, vapors, stressed test organisms, etc. Within the acute, 96-hour tests ten percent control mortality may be present due to inherent biological factors. Any increase above this is considered to be due to conditiions of the test or the test organisms. The ten percent mortality figure is representative for a wide variety of organisms, including both fish and invertebrates (ASTM 1979) and is generally utilized for 96-hour testing. Some of this mortality in invertebrates may be associated with injury during handling.

In an analysis of thirteen life cycle tests which utilized M. bahia (Nimmo, unpublished laboratory data; Gulf Breeze EPA Laboratory), control mortality over the testing period (20-28 days) ranged from 0-31 percent. The mean control mortality of these studies was 11 percent, with 34 percent of the test results greater than the 11 percent. Control mortality of 20 percent in the chronic test will be considered to be due to conditions of the test or the test organisms.

h. Carriers

Carriers can effect test organisms and can possibly alter the form of the test substance in water. For these reasons it is preferable to avoid the use of carriers in toxicity tests unless absolutely necessary to dissolve the test substance. Since carriers can stress or adversely

effect test organisms, the amount of carrier should be kept to a minimum. Recommended maxima are 0.5 ml/l in static tests and 0.1 ml/l in flow-through tests (APHA 1975).

Triethlyene glycol has been found to exert the least influence on mysid response to test substances of several carriers that have been used. Acetone and ethanol have a stronger tendency to reduce the surface tension of the water, and therefore, decrease oxygen saturation (Veith and Comstock 1975, Krugel et al. 1978, APHA 1975).

i. Randomization

The test chamber position in the testing area and assignment of mysids to test chambers are randomized to prevent conscious or unconscious biases from being introduced. These biases can be in environmental conditions and distribution, dilutor system function, etc.

4. Environmental Conditions

a. Dissolved Oxygen

In flow-through testing, large variations in flow rates to aquaria will result in undesirable differences in exposure and test conditions between aquaria. Parameters such as dissolved oxygen and test substance concentration can decrease more rapidly in aquaria with low flow rates; similarly metabolic products can build-up under these conditions. Proper dilution water filtration and mesh size of retention chambers are of utmost importance in maintaining flow rate of test solutions and exchange within retention chambers. Previous studies (Nimmo et al. 1977, USEPA 1978) have found that a flow rate which allows five test chamber volume changes per 24 hours is adequate to obtain necessary conditions for mysid testing.

b. Light

A 14-hour light and 10-hour dark photoperiod is recommended in an effort to approximate representative natural conditions under which M. bahia is found and thus reduce test-related stress. If used, this photoperiod should remain constant throughout culturing, holding and testing, as any deviations could effect test results. Mysids appear very sensitive to light changes and intensity. At very high intensities swimming is inhibited, with mysids sinking to the tank bottom. In very dim light, shoaling behavior is disrupted (Steven 1961). With use of a light-dark regime a transition period is recommended. Mysids are known to react to sudden light changes by jumping out of the water. Gradual transition will avoid mortality caused by mysids "sticking" to test chamber walls.

c. Temperature and Salinity

Test temperature and salinity choices (25°± 2°C and 20 ± 3 °/oo, respectively) were made after review of reports on the habitat characteristics of M. bahia (Nimmo et al. 1977, Price 1978). These temperature and salinity ranges were also found to produce the greatest reproductive success in laboratory cultures of M. bahia (Nimmo et al. 1977, USEPA 1978). Furthermore, minimizing variability in testing conditions through specific temperature and salinity values allows greater comparability of interlaboratory test results and the development of a comparative toxicology data base. An acceptable method for maintaining desired temperature and salinity ranges in flow-through bioassays with marine organisms is described in Bahner and Nimmo (1975).

C. Reporting

A coherent theory of the dose-response relationship, on which the acute toxicity tests are based, was introduced by Bliss (1935), and is widely accepted today. This theory is based on four assumptions:

(1) Response is a positive function of dosage, i.e. it is expected that increasing treatment rates should produce increasing responses.

(2) Randomly selected animals are normally distributed with respect to their response to a toxicant.

(3) Due to homeostasis, response magnitudes are proportional to the logarithm of the dosage, i.e. it takes geometrically increasing dosages (stresses) to produce arithmetically increasing responses (strains) in test animal populations.

(4) In the case of a direct dosage of animals, their resistance to effects is proportional to body mass. Stated another way, the treatment needed to produce a given response is proportional to the size of the animals treated.

The concentration-response curve, where percent mortality is plotted as a function of the logarithm of test solution concentration, can be interpreted as a cumulative distribution of tolerance within the population (Hewlett and Plackett 1979). Experiments designed to measure tolerance directly (Bliss 1944) have shown that tolerance is lognormally distributed within an experimental population in most cases. Departures from the lognormal pattern of distribution are generally associated with mixtures of very susceptible and very resistant individuals within a

population (Hewlett and Plackett 1979). In addition, mixtures of toxicants can produce tolerance curves which deviate significantly from the lognormal pattern (Finney 1971).

If tolerances are lognormally distributed within the experimental population, the resulting concentration-response curve will be sigmoidal in shape, resembling a logistic population curve (Hewlett and Plackett 1979). While estimates for the mean lethal dose can be made directly from the dose response curve, a linear transformation often is possible, using probit (Bliss 1934, Finney 1971) or logit (Hewlett and Plackett 1979) transformations.

Once the mortality data have been transformed, a straight line can be fitted to the points by a least squares linear regression equation and confidence limits can be determined for predicted mortality values. An additional advantage is that the significance of the slope of the regression line can be determined (Draper and Smith 1976).

While the mean lethal dose (LC50) can be estimated graphically from the linearized dose-response curve (APHA 1975), other techniques are preferable since the graphical method does not permit the calculation of confidence limits.

The probit method (Finney 1971), which is recommended in the acute toxicity test guideline, uses the probit transformation and the maximum likelihood curve-fitting technique. Other appropriate tests used in data reduction include the modified probit method of Litchfield and Wilcoxon (1949), the logit method (Ashton 1972) and the

moving average method (Thompson 1947).

If there are no partial kills in an experiment, determination of the concentration-response curve is not possible. In situations where there are no partial kills, the binominal test (Siegel 1956) can be used to estimate the LC50 and confidence limits around the LC50 value (Stephan 1977).

If concentration-response data are plotted for each 24-hour interval throughout the test, the LC50 determined from each curve can be plotted as a function of time, yielding a time-acute toxicity curve (APHA 1975). This curve approaches a line parallel to the time axis asymptotically, indicating a constant or threshold value for LC50. The absence of a threshold LC50 may indicate the need for an acute test of longer duration.

The statistical tests recommended for analyses of mysid life-cycle data (mortality, body lengths of males and females and young per female) were chosen to permit as complete an interpretation of the quantifiable data as possible. Under most conditions, the analysis of variance (ANOVA) is a powerful statistical test allowing the determination of significant differences between treatment means through incorporation of data variability. This statistical examination is especially important in biological experimentation due to the presence of many sources of inherent variability. In previous chronic mysid testing, Nimmo et al. (In press b) employed the analysis of variance with subsequent comparisons between means utilizing Student-New-Kuels, Duncan's, Dunnett's, or Bonferron's tests. Futhermore, the use of analysis of variance (ANOVA)

and mean separation tests have been employed in mysid testing at the EPA Environmental Research Laboratory at Gulf Breeze, Florida, and have given consistent results under the experimental conditions stated in the test guideline document.

III. Economic Aspects

The Agency awarded a contract to Enviro Control, Inc. to provide us with an estimate of the cost for performing static and flow-through acute toxicity tests and flow-through chronic toxicity tests. Enviro Control supplied us with two estimates; a protocol estimate and a laboratory survey estimate.

Protocol Estimates

	range	mean
Acute (static and flow through)	$ 322-$ 965	$ 643
Chronic	$1653-$4959	$3306

These estimates were prepared by separating the guidelines into individual tasks and estimating the hours used to accomplish each task. Hourly rates were then applied to yield a total direct labor charge. An overhead rate of 115 percent, other direct costs ($50-acute, $415-chronic), a general and administrative rate of 10 percent and a fee of 20 percent were then added to the direct labor charge to yield the final estimate.

Laboratory Survey Estimates

	range	mean
Acute (static and flow through)	$ 340-$ 1250	$ 743
Chronic	-	$3000

The laboratory survey estimates were compiled from three laboratories for the acute guideline and one laboratory for the chronic guideline.

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EG-5
August, 1982

OYSTER ACUTE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
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OYSTER ACUTE TOXICITY TEST

(a) Purpose. This guideline will be used in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA)(Pub.L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 et. seq.). This guideline prescribes tests to be used to develop data on the acute toxicity of chemicals to Eastern oysters, Crassostrea virginica (Gmelin). The United States Environmental Protection Agency (USEPA) will use data from these tests in assessing the hazard of a chemical to the environment.

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

(1) "Acute toxicity" is the discernible adverse effects induced in an organism within a short period of time (days) of exposure to a chemical. For aquatic animals this usually refers to continuous exposure to the chemical in water for a period of up to four days. The effects (lethal or sublethal) occurring may usually be observed within the period of exposure with aquatic organisms. In this test guideline, shell deposition is used as the measure of toxicity.

(2) "EC 50" is that experimentally derived concentration of a chemical in water that is calculated to induce shell deposition 50 percent less than that of the controls in a test batch of organisms during continuous exposure within a particular exposure period which should be stated.

(3) "Shell deposition" is the measured length of shell growth that occurs between the time the shell is ground at test initiation and test termination 96 hours later.

(4) "Umbo" means the narrow end (apex) of the oyster shell.

(5) "Valve height" means the greatest linear dimension of the oyster as measured from the umbo to the ventral edge of the valves (the farthest distance from the umbo).

(c) Test procedures--(1) Summary of the test. (i) The water solubility and the vapor pressure of the test chemical should be known. Prior to testing, the structural formula of the test chemical, its purity, stability in water and light, n-octanol/water partition coefficient, and pK_a values should be known prior to testing. The results of a biodegradability test and the method of analysis for the quantification of the chemical in water should also be known.

(ii) For chemicals with limited solubility under the test conditions, it may not be possible to determine an EC 50. If it is observed that the stability or homogeneity of the test

chemical cannot be maintained, then care should be taken in the interpretation of the results and a note made that these results may not be reproducible.

(iii) Test chambers are filled with appropriate volumes of dilution water. The flow of dilution water through each chamber is adjusted to the rate desired. The test chemical is introduced into each test chamber and the flow-rate adjusted to establish and maintain the desired concentration in each test chamber. Test oysters which have been acclimated and prepared by grinding away a portion of the shell periphery are randomly introduced into the test and control chambers. Oysters in the test and control chambers are observed daily during the test for evidence of feeding or unusual conditions, such as shell gaping, excessive mucus production or formation of fungal growths in the test chambers. The observations are recorded and dead oysters removed. At the end of 96 hours the increments of new shell growth are measured in all oysters. The concentration-response curve and EC 50 value for the test chemical are developed from these data.

(2) [Reserved]

(3) Range-finding test. A range-finding test should be conducted to establish test chemical concentrations for the definitive test. The test is conducted in the same way as the

definitive test except a widely spaced chemical concentration series (i.e. log-interval) is used.

(4) Definitive test. (i) Oysters which meet condition criteria (age, size, reproductive status, health) and which have been acclimated to test conditions should have approximately 3 to 5 mm of the shell periphery, at the rounded (ventral) end, ground away with a small electric disc grinder or other appropriate device, taking care to uniformly remove the shell rim to produce a smooth, rounded blunt profile. The oyster's valves should be held together tightly during grinding to avoid vibrating the shell and injuring the adductor muscle. Oysters of which so much of the shell rim has been removed that an opening into the shell cavity is visible should not be used.

(ii) It is desirable to have shell growth values for the low and high concentrations relatively close to, but different from, 0 and 100 percent. Therefore, the range of concentrations to which the oysters are exposed should be such that in 96 hours relative to the controls, very little shell growth occurs in oysters exposed to the highest concentration and shell growth is slightly less than controls at the lowest concentration. Oysters in the remaining concentrations should have increments of shell growth, such that ideally, the concentration producing 50 percent shell growth relative to the controls is bracketed with at least

one concentration above and one below it.

(iii) The test should be carried out without adjustment of pH unless there is evidence of marked change in the pH of the solution. Then it is advised that the test be repeated with pH adjustment to that of the dilution water and the results reported.

(iv) The test begins when at least 20 prepared oysters are placed in each of the test chambers containing the appropriate concentrations of test substance and controls. The steady-state flows and test chemical concentrations should be documented. At least 5 test chemical concentrations should be used. The dilution factor between concentrations should not exceed 1.8.

(v) The distribution of individual oysters among the test chambers should be randomized. The oysters should be spread out equidistantly from one another so that the entire test chamber is used. The oysters should also be placed with the left (cupped) valve down and the open, unhinged ends all oriented in the same direction facing the incoming flow of test solution.

(vi) The oysters are inspected at least after 24, 48, 72 and 96 hours. Oysters are considered dead if touching of the gaping shell produces no reaction. Dead oysters are removed when observed and mortalities are recorded. Observations at three hours and six hours are also desirable.

(vii) Shell growth is the primary criterion used in this test guideline to evaluate the toxicity of the test chemical. Shell growth increments in all oysters should be measured after 96 hours exposure. Record the length of the longest "finger" of new shell growth to the nearest 0.5 mm. Oysters should be handled very gently at this stage to prevent damage to the new shell growth.

(viii) Records should be kept of visible abnormalities such as loss of feeding activity (failure to deposit feces), excessive mucus production (stringy material floating suspended from oysters), spawning or appearance of shell (closure or gaping).

(ix) The criteria for a valid definitive test are:

(A) The mortality in the controls should not exceed 10 percent at the end of the test.

(B) The dissolved oxygen concentration should be at least 60 percent of air saturation throughout the test.

(C) Oysters should not spawn during test. If they do the test should be repeated with prespawn oysters.

(D) There should be evidence that the concentration of the substance being tested has been satisfactorily maintained (e.g., within 80 percent of the nominal concentration) over the test period. The total concentration of test substance (i.e. both dissolved and suspended undissolved particulates) should be

measured; (1) in each chamber at 0-hour, (2) in each chamber at 96-hours and (3) in at least one appropriate chamber whenever a malfunction is detected in any part of the test chemical delivery system.

(E) Dissolved oxygen, temperature, salinity and pH measurements should be made at the beginning of the test, at 48 hours, and at the end of the test in the control chambers and in those test chambers containing the highest, lowest and a middle concentration of the test substance.

(5) Test results. (i) At the end of the test, a one-way analysis of variance followed with an appropriate ad hoc test (the studentized Neuman-Keul's or Duncan's multiple range tests; or Dunnetts' or Williams' pairwise comparison tests) should be conducted on the oyster shell deposition test data. The probit transformation should then be applied to the response variable and then regressed, using least squares regression, on dose or log-dose. An F Test for linearity should be conducted to determine whether the chosen regression technique adequately describes the experimental data.

(ii) Calculate the ratio of the mean shell growth for each group of test oysters (exposed to each of the test chemical concentrations) to the mean shell growth of the group of control oysters. From these data the concentration-response curve is

drawn and an EC 50 along with the 95 percent confidence limits on the value are determined from the curves. The mean measured concentration of test chemical should be used to calculate the EC 50 and to plot the concentration-response curve.

(6) [Reserved]

(d) Test conditions--(1) Test species--(i) Selection. (A) The Eastern oyster, Crassostrea virginica, should be used as the test organism.

(B) Oysters used in the same test should be 30 to 50 millimeters in valve height and should be as similar in age and/or size as possible to reduce variability. The standard deviation of the valve height should be less than 20 percent of the mean.

(C) Oysters used in the same test should be from the same source and from the same holding and acclimation tank(s).

(D) Oysters should be in a prespawn condition of gonadal development prior to and during the test as determined by direct or histological observation of the gonadal tissue for the presence of gametes.

(ii) Acquisition. Oysters may be cultured in the laboratory, purchased from culture facilities or commercial harvesters, or collected from a natural population in an unpolluted area free from epizootic disease.

(iii) Acclimation. (A) Oysters should be attended to immediately upon arrival. Oyster shells should be brushed clean of fouling organisms and the transfer of the oysters to the holding water should be gradual to reduce stress caused by differences in water quality characteristics and temperature. Oysters should be held for at least 12 to 15 days before testing. All oysters should be maintained in water of the quality to be used in the test for at least seven days before they are used.

(B) During holding, the oysters should not be crowded and the dissolved oxygen concentration should be above 60 percent saturation. The temperature of the holding water should be the same as that used for testing. Holding tanks should be kept clean and free of debris. Cultured algae may be added to dilution water sparingly, as necessary to support life and growth and such that test results are not affected as confirmed by previous testing.

(C) Oysters should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible.

(D) A batch of oysters is acceptable for testing if the percentage mortality over the seven day period prior to testing is less than five percent. If the mortality is between 5 and

10%, acclimation should continue for seven additional days. If the mortality is greater than 10%, the entire batch of oysters should be rejected. Oysters should not be used which appear diseased or otherwise stressed. Oysters infested with mudworms (Polydora sp.), boring sponges (Cliona cellata) or which have cracked, chipped, bored, or gaping shells should not be used.

(2) Test facilities--(i) Apparatus. (A) In addition to normal laboratory equipment, an oxygen meter, equipment for delivering the test chemical, adequate apparatus for temperature control, and test tanks made of chemically inert material are needed.

(B) Constant conditions in the test facilities should be maintained as much as possible throughout the test. The preparation and storage of the test material, the holding of the oysters and all operations and tests should be carried out in an environment free from harmful concentrations of dust, vapors and gases and in such a way as to avoid cross-contamination. Any disturbances that may change the behavior of the oysters should be avoided.

(ii) Dilution water. A constant supply of good quality unfiltered seawater should be available throughout the holding, acclimation and testing periods. Natural seawater is recommended, although artificial seawater with food added may be

used. In either case, to ensure each oyster is provided equal amounts of food, the water should come from a thoroughly mixed common source and should be delivered at a flowrate of at least one and preferably five liters per hour per oyster. The flowrate should be ± 10 percent of the nominal flow. A dilution water is acceptable if oysters will survive and grow normally for 14 days without exhibiting signs of stress; i.e. excessive mucus production (stringy material floating suspended from oysters), lack of feeding, shell gaping, poor shell closing in response to prodding, or excessive mortality. The dilution water should have a salinity in excess of 12 parts per thousand, and should be similar to that in the environment from which the test oysters originated. A natural seawater should have a weekly range in salinity of less than 10 parts per thousand and a monthly range in pH of less than 0.8 units. Artificial seawater salinity should not vary more than 2 parts per thousand nor more than 0.5 pH units. Oysters should be tested in dilution water from the same origin.

(3) Test parameters (i) Carriers. Stock solutions of substances of low aqueous solubility may be prepared by ultrasonic dispersion or, if necessary, by use of organic solvents, emulsifiers or dispersants of low toxicity to oysters. When such carriers are used the control oysters should

be exposed to the same concentration of the carrier as that used in the highest concentration of the test substance. The concentration of such carriers should not exceed 0.1 ml/l.

(ii) Dissolved oxygen. The dissolved oxygen concentrations should be at least 60 percent of the saturation value and should be recorded daily.

(iii) Loading. The loading rate should not crowd oysters and should permit adequate circulation of water while avoiding physical agitation of oysters by water current.

(iv) Temperature. The test temperature is 20°C ± 1°C. Temporary fluctuations (less than 8 hours) within 15°C to 25°C are permissible. Temperature should be recorded continuously.

(v) pH. The pH should be recorded twice weekly in each test chamber.

(e) Reporting. In addition to the reporting requirements prescribed in Part 792--Good Laboratory Practice Standards, the report should contain the following:

(1) The source of the dilution water, the mean, standard deviation and range of the salinity, pH, temperature, and dissolved oxygen during the test period.

(2) A description of the test procedures used (e.g. the flow-through system, test chambers, chemical delivery system,

aeration, etc.).

(3) Detailed information about the oysters used, including the age and/or size (i.e. height), source, history, method of confirmation of prespawn condition, acclimation procedures, and food used.

(4) The number of organisms tested, the loading rate, and the flowrate.

(5) The methods of preparation of stock and test solutions, and the test chemical concentrations used.

(6) The number of dead and live test organisms, the percentage of organisms that died, and the number that showed any abnormal effects in the control and in each test chamber at each observation period.

(7) The 96-hour shell growth measurements of each oyster; the mean, standard deviation and range of the measured shell growth at 96 hours of oysters in each concentration of test substance and control.

(8) The calculated 96 hour EC 50 and its 95 percent confidence limits and the statistical methods used to calculate these values.

(9) When observed, the 96 hour observed no-effect concentration (the highest concentration tested at which there were no mortalities, abnormal behavioral or physiological effects

and at which shell growth did not differ from controls).

(10) A graph of the concentration-response curve based on the 96 hour chemical concentration and shell growth measurements upon which the EC 50 was calculated.

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

(12) Any incidents in the course of the test which might have influenced the results.

(13) A statement that the test was carried out in agreement with the prescriptions of the test guideline given above (otherwise a description of any deviations occuring).

EG-6
August, 1982

OYSTER BIOCONCENTRATION TEST

OFFICE OF TOXIC SUBSTANCES
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OYSTER BIOCONCENTRATION TEST

(a) Purpose. This guideline is to be used for assessing the propensity of chemical substances to bioconcentrate in tissues of estuarine and marine molluscs. This guideline describes a bioconcentration test procedure for the continuous exposure of Eastern oysters (Crassostrea virginica) to a test substance in a flow-through system. The United States Environmental Protection Agency (USEPA) will use data from this test in assessing the hazard a chemical may present to the environment.

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

(1) "Acclimation" is the physiological compensation by test organisms to new environmental conditions (e.g., temperature, salinity, pH).

(2) "Bioconcentration" is the net accumulation of a chemical directly from water into and onto aquatic organisms.

(3) "Bioconcentration factor (BCF)" is the quotient of the concentration of a test chemical in tissues of aquatic organisms at or over a discrete time period of exposure divided by the concentration of test chemical in the test water at or during the same time period.

(4) "Depuration" is the elimination of a test chemical from a test organism.

(5) "Depuration phase" is the portion of a bioconcentration test after the uptake phase during which the organisms are in flowing water to which no test chemical is added.

(6) "EC 50" is that experimentally derived concentration of a chemical in water that is calculated to induce shell deposition 50 percent less than that of the controls in a test batch of organisms during continuous exposure within a particular period of exposure (which should be stated).

(7) "Loading" is the ratio of the number of oysters to the volume (liters) of test solution passing through the test chamber per hour.

(8) "Steady-state" is the time period during which the amounts of test chemical being taken up and depurated by the test oysters are equal, i.e., equilibrium.

(9) "Steady-state bioconcentration factor" is the mean concentration of the test chemical in test organisms during steady-state divided by the mean concentration of the test chemical in the test solution during the same period.

(10) "Umbo" is the narrow end (apex) of the oyster shell.

(11) "Uptake" is the sorption of a test chemical into and onto aquatic organisms during exposure.

(12) "Uptake phase" is the initial portion of a bioconcentration test during which the organisms are exposed to the test solution.

(13) "Valve height" is the greatest linear dimension of the oyster as measured from the umbo to the ventral edge of the valves (the farthest distance from the umbo).

(c) Test procedures--(1) Summary of the test. Oysters are continuously exposed to a minimum of one constant, sublethal concentration of a test chemical under flow-through conditions for a maximum of 28 days. During this time, test solution and oysters are periodically sampled and analyzed using appropriate methods to quantify the test chemical concentration. If, prior to day 28, the tissue concentrations of the chemical sampled over three consecutive sampling periods have been shown to be statistically similar (i.e., steady-state has been reached), the uptake phase of the test is terminated, and the remaining oysters are transferred to untreated flowing water until 95 percent of the accumulated chemical residues have been eliminated, or for a maximum depuration period of 14 days. The mean test chemical concentration in the oysters at steady-state is divided by the mean test solution concentration at the same time to determine the bioconcentration factor (BCF). If steady-state is not reached during 28 days of uptake, the steady-state BCF should be

calculated using non-linear parameter estimation methods.

(2) [Reserved]

(3) Range-finding test. The oyster acute toxicity test is used to determine the concentration levels to be used in the oyster bioconcentration test.

(4) Definitive test. (i) The following data on the test chemical should be known prior to testing:

(A) Solubility in water.

(B) Stability in water.

(C) Octanol-water partition coefficient.

(D) Acute toxicity (e.g. propensity to inhibit shell deposition) to oysters.

(E) The validity, accuracy and minimum detection limits of selected analytical methods.

(ii) At least one or more concentrations should be tested to assess the propensity of the compound to bioconcentrate. The concentrations selected should not stress or adversely affect the oysters and should be less than one-tenth the EC 50 determined in either the range-finding or 96-hour definitive test in the Oyster Acute Toxicity Test Guideline (USEPA 1981). The test concentration should be less than the solubility limit of the test substance in water and should be close to the potential or expected environmental concentration. The limiting factor of how

low one can test is based on the detection limits of the analytical methods. The concentration of the test material in the test solution should be at least ten times greater than the detection limit in water.

(iii) If it is desirable to document that the potential to bioconcentate is independent of the test chemical concentration, at least two concentrations should be tested which are at least a factor of 10 apart.

(iv) To determine the duration of this test, an estimation of the uptake phase should be made prior to testing based upon the water solubility or octanol-water partition coefficient of the test chemical. This estimate should also be used to designate a sampling schedule.

(v) The following criteria should be met for a valid test:

(A) If it is observed that the stability or homogeneity of the test chemical cannot be maintained, then care should be taken in the interpretation of the results and a note made that these results may not be reproducible.

(B) The mortality in the controls should not exceed 10 percent at the end of the test.

(C) The dissolved oxygen concentration should be > 60 percent of saturation throughout the test.

(D) There should be evidence that the concentration of the

chemical being tested has been satisfactorily mainained (e.g. within 80 percent of the nominal concentration) over the test period.

(E) Results are invalid and the test should be repeated if the oysters spawn during the test.

(F) Temperature variations from 20°C should be held to a minimum.

(vi) The following methodology should be followed:

(A) The test should not be started until the test chemical delivery system has been observed to be functioning properly and the test chemical concentrations have equilibrated (i.e. the concentration does not vary more than 20 percent). Analyses of two sets of test solution samples taken prior to test initiation should document this equilibrium. At initiation (time 0), test solution samples should be collected immediately prior to the addition of oysters to the test chambers.

(B) The appropriate number of oysters should be brushed clean and should be randomly distributed to each test chamber. The oysters should be spread out equidistant from one another and placed with the left (cupped) valve down and the unhinged ends (opposite from umbo) all oriented in the same direction facing the incoming flow.

(C) Oysters should be exposed to the test chemical during

the uptake phase until steady state has been reached or for a maximum of 28 days. The uptake phase should continue for at least 4 days. Then the remaining oysters should be transferred to untreated flowing water and sampled periodically to determine if depuration of the test chemical occurs. Every test should include a control consisting of the same dilution water, conditions, procedures, and oysters from the same group used in the test, except that none of the test chemical is added. If a carrier is present in the test chamber, a separate carrier control is required.

(D) Oysters should be observed (and data recorded) at least daily for feeding activity (deposition of feces) or any unusual conditions such as excessive mucus production (stringy material floating suspended from oysters), spawning, or appearance of shell (closure or gaping). If gaping is noted, the oyster(s) should be prodded. Oysters which fail to make any shell movements when prodded are to be considered dead, and should be removed promptly with as little disturbance as possible to the test chamber(s) and remaining live oysters.

(E) For oysters sampled, careful examination of all the tissues should be made at the time of shucking for any unusual conditions, such as a watery appearance or differences in color from the controls.

(F) Observations on compound solubility should also be recorded. These include the appearance of surface slicks, precipitates, or material adsorbing to the test chamber.

(vii) Sampling. (A) At each of the designated sampling times, triplicate water samples and enough oysters should be collected from the test chamber(s) to allow for tissue analyses of at least four oysters. The concentration of test chemical should be determined in a minimum of 4 oysters analyzed individually at each sampling period. If individual analysis is not possible, due to limitations of the sensitivity of the analytical methods, then pairs, triplicates or more oysters may be pooled to constitute a sample for measurement. A similar number of control oysters should also be collected at each sample point, but only those collected at the first sampling period and weekly thereafter, should be analyzed. Triplicate control water samples should be collected at the time of test initiation and weekly thereafter. Test solution samples should be removed from the approximate center of the water column.

(B) At each sampling period the appropriate numbers of oysters are removed and treated as follows:

(1) The valve height of each oyster should be measured.

(2) Oysters should be shucked as soon as practical after removal and should never be refrigerated or frozen in the

shell. The shell should be opened at the hinge, the adductor muscle severed and the top valve removed. The remaining adductor muscle should be severed where it attaches to the lower valve and the entire oyster removed.

(3) The shucked oysters should then be drained three minutes, blotted dry, weighed and analyzed immediately for the test chemical. If analyses are delayed, the shucked oysters should be wrapped individually in aluminum foil (for organic analysis) or placed in plastic or glass containers (for metal analysis) and frozen.

(C) If a radiolabelled test compound is used, a sufficient number of oysters should also be sampled at termination to permit identification and quantitation of any major (greater than 10 percent of parent) metabolites present. It is crucial to determine how much of the activity present in the oyster is directly attributable to the parent compound.

(5) Test results (i) Steady-state has been reached when the mean concentrations of test chemical in whole oyster tissue for three consecutive sampling periods are statistically similar (F test, P=0.05). A BCF is then calculated by dividing the mean tissue residue concentration during steady-state by the mean test solution concentration during this same period. A 95 percent confidence interval should also be derived from the BCF. This

should be done by calculating the mean oyster tissue concentration at steady-state (X_o) and its 97.5 percent confidence interval $X_o \pm t$ (S.E.) where t is the t statistic at P=0.025 and S.E. is the one standard error of the mean. This calculation would yield lower and upper confidence limits (L_o and U_o). The same procedure should be used to calculate the mean and 97.5 percent confidence interval for the test solution concentrations at steady-state, Xs ± t(S.E.), and the resulting upper and lower confidence limits (L_s and U_s). The 95 percent confidence interval of the BCF would then be between L_o/U_s and U_o/L_s. If steady-state was not reached during the maximum 28 day uptake period, the maximum BCF should be calculated using the mean tissue concentration from that and the previous sampling day. An uptake rate constant should then be calculated using appropriate techniques. This rate constant is used to estimate the steady-state BCF and the time to steady-state.

(ii) If 95 percent elimination has not been observed after 14 days depuration then a depuration rate constant should also be calculated. This rate constant is used to estimate the time to 95 percent elimination.

(iii) Oysters used in the same test should be 30 to 50 millimeters in valve height and should be as similar in age and/or size as possible to reduce variability. The standard

deviation of the height should be less than 20 percent of the mean (N=30).

(6) Analytical measurements. (i) All samples should be analyzed using USEPA methods and guidelines whenever feasible. The specific methodology used should be validated before the test is initiated. The accuracy of the method should be measured by the method of known additions. This involves adding a known amount of the test chemical to three water samples taken from an aquarium containing dilution water and a number of oysters equal to that to be used in the test. The nominal concentration of these samples should be the same as the concentration to be used in the test. Samples taken on two separate days should be analyzed. The accuracy and precision of the analytical method should be checked using reference or split samples or suitable corroborative methods of analysis. The accuracy of standard solutions should be checked against other standard solutions whenever possible.

(ii) An analytical method should not be used if likely degradation products of the test chemical, such as hydrolysis and oxidation products, give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test. Atomic absorption spectrophotometric methods for metal and gas chromatographic

methods for organic compounds are preferable to colorimetric methods.

(iii) In addition to analyzing samples of test solution at least one reagent blank should also be analyzed when a reagent is used in the analysis.

(iv) When radiolabelled test compounds are used, total radioactivity should be measured in all samples. At the end of the uptake phase, water and tissue samples should be analyzed using appropriate methodology to identify and estimate the amount of any major (at least 10 percent of the parent compound) degradation products or metabolites that may be present.

(d) Test conditions--(1) Test species. (i) The Eastern oyster, Crassostrea virginica, should be used as the test organism.

(ii) Oysters used in the same test should be 30 to 50 millimeters in valve height and should be as similar in age and/or size as possible to reduce variability. The standard deviation of the valve height should be less than 20 percent of the mean.

(iii) Oysters used in the same test should be from the same source and from the same holding and acclimation tank(s).

(iv) Oysters should be in a prespawn condition of gonadal development prior to and during the test as determined by direct

or histological observation of the gonadal tissue for the presence of gametes.

(v) Oysters may be cultured in the laboratory, purchased from culture facilities or commercial harvesters, or collected from a natural population in an unpolluted area free from epizootic disease.

(vi) The holding and acclimation of the oysters should be as follows:

(A) Oysters should be attended to immediately upon arrival. Oyster shells should be brushed clean of fouling organisms and the transfer of the oysters to the holding water should be gradual to reduce stress caused by differences in water quality characteristics and temperature. Oysters should be held for at least 12 to 15 days before testing. All oysters should be maintained in water of the quality to be used in the test for at least seven days before they are used.

(B) During holding, the oysters should not be crowded and the dissolved oxygen concentration should be above 60 percent saturation. The temperature of the holding waters should be the same as that used for testing. Holding tanks should be kept clean and free of debris. Cultured algae may be added to dilution water sparingly, as necessary to support life and growth, such that test results are not affected, as confirmed by previous

testing. Oysters should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible.

(C) A batch of oysters is acceptable for testing if the percentage mortality over the seven day period prior to testing is less than five percent. If the mortality is between 5 and 10 percent, acclimation should continue for seven additional days. If the mortality is greater than 10 percent, the entire batch of oysters should be rejected. Oysters should not be used which appear diseased or otherwise stressed. Oysters infested with mudworms (Polydora sp.), boring sponges (Cliona cellata) or which have cracked, chipped, bored, or gaping shells should not be used.

(2) Facilities--(i) Apparatus. (A) An oxygen meter, equipment for delivering the test chemical, adequate apparatus for temperature control, test tanks made of chemically inert material and other normal laboratory equipment are needed.

(B) Constant conditions in the test facilities should be maintained as much as possible throughout the test. The preparation and storage of the test material, the holding of the oysters and all operations and tests should be carried out in an environment free from harmful concentrations of dust, vapors and gases and in such a way as to avoid cross-contamination. Any

disturbances that may change the behavior of the oysters should be avoided.

(ii) Dilution water A constant supply of good quality unfiltered seawater should be available throughout the holding, acclimation, and testing periods. Natural seawater is recommended, although artificial seawater with food (algae) added may be used. In either case, to ensure each oyster is provided equal amounts of food, the water should come from a thoroughly mixed common source and should be delivered at a flow rate of at least one, and preferably five liters per hour per oyster. The flowrate should be ± 10 percent of the nominal flow. A dilution water is acceptable if oysters will survive and grow normally over the period in which the test is conducted without exhibiting signs of stress, i.e. excessive mucus production (stringy material floating suspended from oysters), lack of feeding, shell gaping, poor shell closing in response to prodding, or excessive mortality. The dilution water should have a salinity in excess of 12 parts per thousand, and should be similiar to that in the environment from which the test oysters originated. A natural seawater should have a weekly range in salinity of less than 10 parts per thousand and a monthly range in pH of less than 0.8 units. Artificial seawater should not vary more than 2 parts per thousand nor more than 0.5 pH units. Oysters should be tested in

dilution water from the same origin.

(3) Test parameters--(i) Carriers. Stock solutions of substances of low aqueous solubility may be prepared by ultrasonic dispersion or, if necessary, by use of organic solvents, emulsifiers or dispersants of low toxicity to oysters. When such carriers are used, the control oysters should be exposed to the same concentration of the carrier as that used in the highest concentration of the test substance. The concentration of such carriers should not exceed 0.1 ml/l.

(ii) Dissolved oxygen. The dissolved oxygen concentrations should be at least 60 percent of the air saturation value and should be recorded daily.

(iii) Loading. The loading rate should not crowd oysters and should permit adequate circulation of water while avoiding physical agitation of oysters by water current.

(iv) Temperature. The test temperature should be 20°C ± 1°C. Temporary excursions (less than eight hours) within 15°C to 25°C are permissible. Temperature should be recorded continuously.

(v) pH. The pH should be recorded twice weekly in each test chamber.

(e) Reporting. In addition to the reporting requirements prescribed in Part 792--Good Laboratory Practice Standards, the

report should contain the following:

(1) The source of the dilution water, the mean, standard deviation and range of the salinity, pH, temperature and dissolved oxygen during the test period.

(2) A description of the test procedures used (e.g. the flow-through system, test chambers, chemical delivery system, aeration, etc.).

(3) Detailed information about the oysters used, including age, and/or size (i.e. height), weight (blotted dry), source, history, method of confirmation of prespawn condition, acclimation procedures,and food used.

(4) The number of organisms tested, loading rate and flowrate.

(5) The methods of preparation of stock and test solutions and the test chemical concentrations used.

(6) The number of dead and live organisms, the percentage of oysters that died and the number that showed any abnormal affects in the control and in each test chamber at each observation period.

(7) Methods and data records of all chemical analyses of water quality parameters and test chemical concentrations, including method validations and reagent blanks.

(8) Description of sampling, sample storage (if required) and analytical methods of water and tissue analyses for the test chemical.

(9) The mean, standard deviation and range of the concentration of test chemical in the test solution and oyster tissue at each sampling period.

(10) The time to steady-state.

(11) The steady-state or maximum BCF and the 95 percent confidence limits.

(12) The time to 95 percent elimination of accumulated residues of the test chemical from test oysters.

(13) Any incidents in the course of the test which might have influenced the results.

(14) If the test was not done in accordance with the prescribed conditions and procedures, all deviations should be described in full.

(f) References. U.S. Environmental Protection Agency. 1981. Oyster Acute Toxicity Test Guideline, Toxic Substances Control Act, section 4. Office of Pesticides and Toxic Substances, Washington, D.C.: U.S. Environmental Protection Agency.

ES-3
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TECHNICAL SUPPORT DOCUMENT

FOR

OYSTER ACUTE TOXICITY TEST AND BIOCONCENTRATION TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

TABLE OF CONTENTS

I. Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guidelines EG-5 and EG-6 which uses the Eastern oyster ,Crassostrea virginia, to evaluate toxicity and bioconcentration of chemical substances. The Document provides an account of the logic used in the selection of the test methodology, procedures and conditions prescribed in the Test Guidelines. Technical issues and practical considerations relevant to the Test Guidelines are discussed. In addition, estimates of the cost of conducting the tests are provided.

II. Scientific Aspects

A. General

The test guidelines represent a synthesis of testing procedures and the current laboratory practices of various researchers. Increased interest and research in aquatic toxicology and bioconcentration and their use as monitoring tools has led to the need for standardized procedures for testing the sublethal responses of marine and estuarine bivalve molluscs. Mortality testing is not practical because bivalves are able to close their valves and seal themselves off from environmental stress for long periods of time. The results of such tests would be difficult, if not impossible, to interpret. A more useful test is the shell deposition test, which employs concentrations that will produce an adverse effect, but will not cause the animal to close up. The shell deposition test is intended to provide a short-term assessment of the hazard which a test chemical

may present to oysters, and to serve as a range-finding test for the bioconcentration test. Therefore, the test should be of short duration, and be similar in conditions to the bioconcentration test.

Butler et al. (1960) demonstrated that shell growth in juvenile oysters could be employed as a sensitive method for the continuous monitoring of physiological stress occurring in bivalves exposed to various concentrations of pesticides. In Butler's studies, shell growth was used as a measure of reversible inhibitory effect. The advantage of shell growth lies in the ability to use the shell as an ongoing physiological stress monitor without the need for periodic sacrificing of organisms. In addition, the test is rapid, reliable, reproducible and requires no specialized equipment or personnel training.

The method first developed by Butler which utilized shell deposition as a bioassay technique has been successfully employed by numerous researchers (Schuster and Pringle 1969, Tinsman and Maurer 1974, Frazier 1976, Conger et al. 1978, Cunningham 1976, Epifanio and Mootz 1976, Lowe et al. 1972). Epifanio (1979) showed that growth of hard and soft tissues in oysters was closely coupled, as determined by correlation analysis. This indicates that shell deposition serves as a useful indicator of oyster physiological response rather than just as a singular response to calcium carbonate deposition. Studies conducted by Conger et al. (1978) indicated a statistically significant difference ($P \leq 0.001$) in the level of inhibition of shell deposition in oysters which were subjected to 0.25 mg/l cadmium. Schimmel et al. (1976) compared a 50 percent

median shell deposition reduction (EC50) in Crassostrea virginica and the LC50 in five fish species. In this study, shell deposition was at least as sensitive a test as those employing fish. In tests conducted by Butler (1965) the shell deposition test was more sensitive than other acute toxicity tests employing other estuarine organisms. The oyster shell deposition methodology is also described in Standard Methods (APHA 1975), Bioassay Procedures for the Ocean Disposal Permit Program (USEPA 1978) and American Society for Testing and Materials (ASTM 1980).

Interest in bioconcentration began with the discovery that levels of heavy metals in many animals were much higher than in the surrounding water. In oysters, the phenomenon was observed early in this century (Hiltner and Wickman 1919), and elaborated upon by subsequent workers (Galtsoff 1942, Chipman et al. 1958). The increased impacts of hydrocarbons and various organic compounds, such as insecticides, in recent years, led to field studies of bioconcentration by oysters of such chemicals (Stegeman and Teal 1973, Butler 1967, Hansen et al. 1976, Brodtmann 1970).

Laboratory testing of oyster bioconcentration is a relatively recent development. Schimmel et al. (1977), Bahner et al. (1977), Frazier (1979a,b), Hansen et al. (1976), Lee et al. (1978), Parrish et al. (1976), and Stegeman (1974) have all investigated bioconcentration using oysters in controlled laboratory settings. However, the experimental methodology of each investigator was often substantially different.

Other flow-through testing, particularly concerned with sublethal effects on fish and bivalves in the late 1960's

and early 1970's led to a general recognition of the need for standardization in flow-through testing. Several authors proposed methodologies (Sprague 1969, Cairns and Dickson 1973, Esvelt and Connors 1971, Lichatowich et al. 1973, LaRoche et al. 1970). These were synthesized into the bioassay section of Standard Methods (APHA 1975) and sections of the Ocean Disposal Bioassay Manual (Butler and Lowe 1978). However, neither publication considered bioconcentration. Bioconcentration methodologies have emerged only in the last few years, and have been drafted as proposed standards by the American Society for Testing and Materials (ASTM 1980).

The test guidelines adapt, to the extent possible, the procedures of Standard Methods, EPA and ASTM to the specific requirements of the Eastern oyster, Crassostrea virginia Gmelin.

Many industrial chemicals have not been previously tested by standard aquatic bioassay methods and, as a result, cannot be classified as to their toxicological properties or propensity to bioconcentrate.

The oyster shell deposition test provides information on the effects of short-term exposure of the test oysters to the test chemical under controlled conditions (ASTM 1980b). Continuous administration of the test chemical in this 96-hour flow-through system represents a practial simulation of chemical spills of effluent discharges to non-motile organisms which are incapable of avoiding the perturbation (APHA 1975). As such, the oyster shell deposition test is particularly useful for evaluating the short-term toxicity of specific substances or wastes on

marine molluscs. This test is employed primarily as an appropriate range-finding test for the more complex bioconcentration test. As a range-finding test, it provides information on the upper limit of exposure that is not anticipated to cause adverse effects during the bioconcentration test.

Oysters, as filter feeders, can be exposed to relatively large amounts of a potential toxicant. This is because the oyster pumps large volumes of water and removes both living and non-living particulate matter from that water. A potential toxicant can be accumulated in the oyster tissues in concentrations much greater than occur in the ambient water or particulate matter. This accumulation, known as bioconcentration, has been demonstrated for a number of petrochemicals (Anderson and Anderson 1976, Anderson 1978, Bahner et al. 1977, Lee et al. 1978, Stegeman 1974), pesticides (Brodtmann 1970, Butler 1967, Parrish et al. 1976, Schimmel et al. 1977), and metals (Frazier 1975, 1976, 1979 a,b). The contaminated organism can, in turn, pass its body burden of toxicant on to the next trophic level in a concentrated form. Since humans are major consumers of oysters, the potential for oysters to bioconcentrate a potentially toxic substance is of additional concern. In addition, bioconcentration of a substance by oysters may be an indication that the substance is biologically active and could affect other elements of the aquatic system.

The bioconcentration test provides an estimate of that potential. The results of the test can provide a basis for decisions concerning what concentrations, if any, of the test chemical in water may be bioconcentrated to potentially

hazardous concentrations in the aquatic biota.

B. Test procedures

1. Range-Finding Test

a. Acute test

For the oyster acute toxicity test, a range-finding test is recommended to determine the appropriate concentrations of test chemical to be used for a definitive test when the acute toxicity of the substance is unknown or cannot be elucidated from existing toxicity data. This approach should minimize the possibility that an inappropriate concentration series will be utilized in the definitive test and under certain circumstances may even preclude the need to conduct the definitive test. In order to minimize the cost and time required to obtain the requisite data nominal concentrations are permitted, test duration may be shortened, replicates are not required, and other test procedures and conditions are relaxed.

The range-finding test (or other available information) needs to be accurate enough to ensure that dose levels in the definitive test are spaced to result in concentrations above and below the EC50 values for shell deposition. If the substance has no measurable effect at the saturation concentration (at least 1000 mg/l), it is considered relatively non-toxic to oysters and definitive testing is deemed unnecessary. In all cases, the range-finding test is conducted to reduce the expense involved in having to repeat a definitive test because of inappropriate test chemical concentrations.

In the range-finding test, groups of five or more test oysters are exposed to a broad range of concentrations of

the substance. Sufficient concentrations should be tested such that the concentration which inhibits shell deposition by 50 percent of the control organisms can be approximated. The number of concentrations will normally range from 3-6 depending upon the shape of the toxicity curve for that substance and prior knowledge of its approximate toxicity. Only concentrations less than the solubility limit in water are tested.

b. Bioconcentration Test

The oyster acute toxicity test is used as the range-finding test for the oyster bioconcentration test. The concentration of test chemical in the test solution should not stress, irritate, or otherwise adversely effect the organisms during the bioconcentration test. To meet this criteria, the ASTM (1980) recommends that the highest concentration be no more than one-tenth the 96 hour EC_{50} based on reduced shell deposition.

If stress, irritation, or other adverse effects are observed, the bioconcentration test should be repeated at a lower concentration.

In the bioconcentration test, it would be most useful for the hazard and risk assessment processes to use an exposure concentration that approximates the expected or estimated environmental concentration. One should take care, however, that the selected concentration is at least three times above its detection limit and will allow quantification of the residues in tissue. Test concentrations of 1-10 ug/l would be appropxiate for many compounds.

2. Definitive test

a. Acute Test

The specific requirements of the definitive oyster acute toxicity test (USEPA 1980) are the analytical determinations of chemical concentrations, the unbiased selection of oysters for each treatment, the use of controls, the assessment of test validity, and the recording, analysis, and presenta-tion of data. These requirements assure that the chemical concentration - oyster response relationship is accurately known, that chemical effects are not confounded by differential oyster sensitivity, and that the relationships are clearly presented. Reporting the occurrence of such effects as abnormal shell movement and feeding behavior provide qualitative data that further assist the assessment of toxicity.

The results of a definitive test are used to calculate the 96 hour EC50 and the concentration-response relationship of the test chemical and the test oysters. If the concentrations of test chemical which produce no effect, a partial inhibition of shell deposition, and 100 percent inhibition have been determined during the range-finding test, then five or six test chemical concentrations should be sufficient to estimate the appropriate EC50 value in a definitive test. In some cases however, to obtain two partial inhibitions bracketing the 50 percent level, it may be necessary to test 8-10 concentrations.

The slope of the concentration-response curve provides an indication of the range of sensitivity of the test oysters to the test chemical and may allow estimations of lower concentrations that will affect the test organism.

For example, if the slope of the concentration-response curve is very steep, then a slight increase in concentration of the test chemical will affect a much greater portion of the test oysters than would a similar increase if the slope of the curve was very shallow. The slope of the concentration-response curve reveals the extent of sensitivity of the test oysters over a range of concentrations.

The exposure of two or more replicate groups having a minimum of 20 oysters each, to each test chemical concentration is required in the guideline. That minimum is based on an optimum number of test oysters needed for statistical confidence, equipment requirements, and practical considerations of handling the test organisms.

At least two replicates should be included in order to demonstrate the level of precision in the data and indicate the significance of variations. Test chambers holding replicate groups should have no water connections between them. The distribution of test oysters to the test chambers should be randomized to prevent bias from being introduced into the test results.

The exposure time of 96 hours in the oyster acute toxicity test guideline is specified in order to permit a comparison of data developed through the use of this test guideline with the acute toxicity data in the published literature (see Section 1.5) The use of the 96-hour exposure period was proposed initially in 1951 by an aquatic bioassay committee (Doudoroff et al. 1951) and was selected, in large part, as a matter of convenience since it is easily scheduled within the five-day work week. The 96-hour

exposure period is also required in the flowing seawater toxicity test using oysters as a bioassay procedure for the USEPA Ocean Disposal Permit Program (USEPA 1978).

b. Bioconcentration Test

The bioconcentration test guideline recommends that the uptake phase last no more than 28 days and the depuration phase last no more than 14 days for a total maximum test duration of 42 days. This is based on the experience of researchers who have found that, generally speaking, substances are either rapidly taken up or very slowly taken up. Krieger et al. (1979) demonstrated attainment of steady-state for antipyrine uptake in less than 90 minutes using mussels. Schimmel et al. (1978) showed that oysters reached uptake equilibrium with respect to sodium pentachlorophenate in 4 days. On the other hand, Stegeman (1974) postulated that while low molecular weight hydrocarbons are rapidly taken up and released, high molecular weight compounds are taken up and released much more slowly and, in fact, may never be completely eliminated. Hydrocarbons apparently reach equilibrium with the lipid fraction of the animal, so that the physiological state of the organism has a great influence on bioconcentration and depuration.

Because of the role of the lipid fraction in modifying bioconcentration, it is possible to generate estimates of the bioconcentration factor of organic chemicals from a knowledge of their lipophilic nature. Veith et al. (1979) analyzed the correlation between the n-octanol/water partition coefficient (P), a commonly used measure of a substance's lipophilic nature, and the experimentally

derived bioconcentration factor (BCF). They show that the log BCF and the log P are linearly related by the equation:

$$\log BCF = 0.85 \log P - 0.70$$

They suggest that the high correlation of the equation ($r^2 = 0.897$) means that the log BCF can be estimated to within an order of magnitude for substances having a broad range of partition coefficients. Approximately 5 percent of the substances tested had low log BCFs despite high log P, thus falling outside of the general relation. However, as Veith et al. (1979) point out, none of the substances with high BCF values had low log P values. This means that use of the relationship should not lead to an underestimation of the bioconcentration factor.

Chiou et al. (1977) present support for estimating the partition coefficient from the aqueous solubility. Their relationship states that:

$$\log P = 5.00 - 0.670 \log S$$

where S is the aqueous solubility in micromol/liter. They found that the log P values for 34 organic substances ranged from 1.26 (for phenoxyacetic acid) to 6.72 (for 2, 3, 4, 2', 4', 5',-PCB).

On the basis of these known relationships between solubility, lipophilic nature and bioconcentration, Neeley (1978) developed the equations for estimating the times to steady-state. The estimates are based on fish, but are applicable to molluscs. In the bioconcentration test, the

exposure period should be long enough to demonstrate that steady-state has been reached.

Before starting a bioconcentration test, an estimation of the BCF or the time to steady-state should be made in order to avoid running the test for the maximum time period. A summary by Kenaga and Goring (1980) presents data and methods to estimate the BCF. The two most commonly used factors for predicting bioconcentration potential are water solubility and octanol-water partitioning. Water solubility can be determined empirically in the laboratory, or in some cases, taken from the literature (Chiou et al. 1977, Kenaga and Goring 1980). Octanol-water partition coefficients can be determined empirically, estimated by reverse-phase high pressure liquid chromatography according to Veith et al. (1979), calculated according to Leo et al. (1971) or taken from the literature (Chiou et al. 1977, Hanch et al. 1972, Kenaga and Goring, 1980). However, some of the reported data are highly variable and may not be appropriate for use.

An estimate of the time to steady-state (S in hours) can be estimated from the water solubility or octanol-water partition coefficient using the equations developed by ASTM (1980): $S=3.0/\text{antilog}\ (0.431 \log W-2.11)$ or $S=3.0/\text{antilog}\ (-0.414 \log P + 0.122)$ where W = water solubility (mg/l) and P=octanol-water partition coefficient.

Presented below is a summary of data correlating various exposure times to the corresponding estimates of the partition coefficient and BCF.

P	Log P	Log BCF	BCF	S
1,585	3.2	.02	105	2
8,710	3.94	2.65	446	4
33,113	4.52	3.14	1387	7
120,226	5.08	4.62	4150	12
316,228	5.5	4.0	10,000	18
524,807	5.72	4.16	14,521	22
933,254	5.97	4.37	23,686	28

Log BCF was estimated using the equation of Veith et al. (1970) where log BCF=0.85 log P-0.70.

Based on the estimate of the time to steady state, one of the following sampling schemes may be used to generate appropriate data.

Sampling Days

Test Period/	S[a]<4	S4-14	S>15-21	S>21
Exposure				
	1[b]	4[b]	1	1
	6[b]	1	3	3
	1	3	7	7
	2	7	10	10
	3	10	14	14
	4	12	18	21
		14	22	28
Depuration				
	1[b]	1	1	1
	6[b]	2	3	3
	12[b]	4	7	7
	1	6	10	10
				14

a. length estimated time to steady state in days.
b. hours.

There are two methodologies in use today to estimate bioconcentration potential; the kinetic approach and the steady-state approach and both are based on research conducted with fish. Bishop and Maki (1980) and Hamelink (1977) give a review of both. Using the kinetic approach, Bishop and Maki (1980), Branson et al. (1975), Cember et al. (1978) and Krzeminsky (1977) proposed the use of first-order kinetic expressions from relatively short ($\leq$ 5 days) fish exposures, and a subsequent depuration period, to calculate uptake and depuration rate constants. These rate constants are then used to estimate the BCF at the time of apparent steady-state, and the time to 50 percent elimination. The steady-state method, in more widespread use, exposes fish for a longer period of time until steady-state in the tissue is experimentally observed (Barrows et al. 1980, Bishop and Maki 1980, Veith et al. 1979) and continues with a depuration phase until 50percent or 95 percent elimination has been observed. The estimation of bioconcentration using the kinetic approach cannot account and adjust for changes in the rates of uptake and depuration such as those observed by Barrows et al. (1980) and Melancon and Lech (1979). The use of the kinetic approach also requires access to a sophisticated computer system, apparatus not readily available to many laboratories.

Although Bishop and Maki (1980) and Branson et al. (1975) have shown excellent agreement between estimates of bio-concentration factors for some compounds using both approaches, the agency has recommended a modified steady-state method for determination of bioconcentration. The empirical nature of the data, the relative ease with which

the test can be performed and the number of researchers and laboratories that have performed such tests make this test more appropriate at this time. As the data base for comparisons of BCFs between the two methods grows, the kinetic approach may become more useful and valuable. Under TSCA the Agency is required to review all test guidelines annually, and in the future the Agency will consider adopting the kinetic approach.

3. Analytical

a. Water Quality Analysis

Measurement of certain water quality parameters of the dilution water such as dissolved oxygen, temperature, salinity and pH is important. Quantification of these parameters at the beginning, during, and at the end of the exposure period for flow-through tests is necessary in order to determine if the water quality varied during the test. If significant variation occurs, the resulting data should be interpreted in light of the estimated toxicity values. A decrease in dissolved oxygen indicates that the flow rate should be increased.

b. Collection of Test Solution Samples

The objective of the recommended sampling procedure is to obtain a representative sample of the test solution for use in measuring the concentration of the test chemical. Although there is mixing in the test chamber, material can concentrate near the sides and bottom of the chamber due to physical or chemical properties of the substance, or to interactions with organic materials associated with the test animals. For this reason, water samples should be taken near the center of the test chamber. The handling and

storage of the samples requires care to prevent the loss of the test chemical from the sample before analysis.

Standardized methods should be used in collecting the samples and performing the analyses to develop chemical and physical data. Appropriate sources for such methodology include, but are not limited to Hedgpeth 1966, Strickland and Parsons 1972, AOAC 1975, APHA 1975, USEPA 1974, and ASTM 1979.

c. Test Chemical Measurement

The actual substance concentration used in the definitive test should be determined with the best available analytical precision. Analysis of stock solutions and test solutions just prior to use will minimize problems with storage (e.g., formation of degradation products, adsorption, transformation, etc.). Nominal concentrations are not adequate for the purposes of the definitive tests. If definitive testing is not required because the substance elicits an insufficient response at the 1000 mg/l level in the range-finding test, the concentration of substance in the test solution should be determined to confirm the actual exposure level. The pH of the test solution should be measured prior to testing to determine if it lies outside of the species' optimal range. This test guideline does not include pH adjustment for the following reasons: the use of acid or base may chemically alter the test chemical making it more or less toxic, the amount of acid or base needed to adjust the pH may vary from one test solution concentration to the next, and the effect the test chemical has on pH may indirectly affect the physiology of the test oysters.

To assess and quantify any possible changes in test chemical concentration, whenever a malfunction of the toxicant delivery system is detected, all potentially affected test chambers should be sampled at that time.

If the measured concentrations of dissolved test chemical are 50 percent more or less than the nominal concentration, steps should be taken to determine the cause for this deviation. A sample of the stock solution as well as influent samples to various test chambers should be analyzed to determine if the reduction in test chemical occurs prior to delivery of the test solution to the aquaria. If results of these analyses indicate that the proper amounts of test substance are entering the test chambers, then the total test chemical concentration should be measured in at least the chambers containing the highest test chemical concentration. These data will give indications if the difference between nominal and measured test concentrations is due to volatilization or degradation of the test chemical, or to insolubility of the test chemical in the dilution water.

If the toxicant delivery system has been properly calibrated and the oysters randomly introduced into each test chamber, the measured differences between replicates at each concentration should be less than 20 percent. If the differences exceed this, the test should be repeated.

The concentrations of test chemical measured after initiation should be within 30 percent of the concentrations measured prior to introduction of the oysters. If the difference exceeds this, the test should be repeated using a higher flow rate.

Use of reliable and validated analytical techniques and methods is essential to the usefulness of the test data in assessing the environmental hazard of the substance. Significant variation in the measured concentrations lessens the value of the toxicity data generated.

4. Test Data

a. Analysis

A coherent theory of the dose-response relationship, on which acute toxicity tests are based, was introduced by Bliss (1935), and is widely accepted today. This theory is based on four assumptions:

(1) Response is a positive function of dosage, i.e. it is expected that increasing treatment rates should produce increasing responses.

(2) Randomly selected animals are normally distributed with respect to their response to a toxicant.

(3) Due to homeostasis, response magnitudes are proportional to the logarithm of the dosage, i.e. it takes geometrically increasing dosages (stresses) to produc arithmetically increasing responses (strains) in test animal populations.

(4) In the case of a direct dosage of animals, their resistance to effects is proportional to body mass. Stated another way, the treatment needed to produce a given response is proportional to the size of the animals treated.

b. Acute Toxicity Test

Oyster shell deposition data have been analyzed by Cunningham (1976) and Schimmel et al. (1976, 1978).

Cunningham (1976) evaluated all shell growth data to the

$P \leq 0.05$ level of confidence using an analysis of variance coupled with a Duncan Multiple-Range test. A detailed summary of the procedure for ranking the means and computing the Duncan Multiple Range (DMR) values is given in Steel and Torrie (1960).

Schimmel et al. (1976) developed an EC50, which is that concentration of toxicant which produces a 50 percent median shell deposition reduction in test oysters as compared to control oysters. During other studies, Schimmel et al. (1978), analyzed oyster shell deposition data by linear regression with probit transformation to determine the EC50 and 95 percent confidence intervals.

Two types of statistical techniques should be employed for analyzing oyster shell deposition data: 1) analysis of variance and 2) linear or non-linear regression. The test design that is assumed is control, carrier control (if solvent carrier is utilized), and five test concentrations giving a total of seven treatments. For each treatment, 20 similar-sized oysters are tested for 96 hours. At the end of the test, each oyster's shell deposition is measured and recorded, giving 20 separate growth rsponses for each treatment.

At this point, it is appropriate to conduct a one-way ANOVA on these data to determine if there is a significant effect on shell deposition due to the treatments (test concentrations). A significant F value (P less than or equal to 0.05) would indicate such an effect and should be following with an appropriate post hoc test (e.g., the studentized Neuman-Keuls' or Duncan's multiple range tests, or Dunnetts' or Williams' pairwise comparison tests). These tests are designed to indicate

which test concentrations caused significant effects. For some situations this information may be all that is necessary; i.e., proof that a statistically significant effect has occured due to the test substance. On the other hand, if the ANOVA shows no significant effect due to treatments, then the criteria requiring effects on both sides of 50 percent will not have been met. If the control and control with carrier are different, then there are severe test problems that should be rectified. Either the solvent is toxic at the concentrations tested, or there is large variability among oysters, probably undiagnosed disease or improper test apparatus.

The second method of analysis to be utilized is regression--either linear with possible data transformations or non-linear least squares. Growth data collected from an oyster shell deposition test is dose-response in which the responses are graded (or continuous) as opposed to quantal (discrete or binomial). Due to this fact, the distribution of measurements at each concentration level is generally assumed to be normally distributed and the response curve is sigmoid (slant S shaped); in the center of the response curve, the curve is typically relatively straight, while at each end of the curve, the curve becomes asymptotic to the 100 percent (control growth or no-effect) and 0 percent (no growth or full-effect) levels. Use of the guideline's proposed test design causes the following to occur: two controls and one no-effect concentration groups 60 oyster growth values at the no-effect end of the rsponse curve. The highest or full-effect concentration groups 20 oyster growth values at the other end of the response curve. That

leaves 20 growth values in each of three treatments to describe the linear central portion of the curve. Linear regression, if used, should depend only on data from the three central treatments, since 0 and 100 percent responses may be far out on the curved ends of the response curve. Improper use of linear regression on data from all treatments will likely overestimate the EC_{50} and widen the associated confidence interval, especially if the highest test concentration was chosen to be very high as compared with the other test concentrations. On the other hand, if all 5 concentrations provide partial response, then simple linear regression on growth data is an appropriate model if the fit is reasonable.

The alternate approaches to straight linear regression are: 1) regular probit analysis regression (using maximum likelihood or minimum), 2) various transformations prior to least squares linear regression, and 3) non-linear regression.

Probit analysis assigns relatively small weights to response values near 0 and 100 percent. This is one of the primary reasons why this analysis is acceptable for use on dose-response data that contain no-effect and full-effect concentrations. Although probits do not exist for 0 and 100 percent effects, they replaced with close estimates and used in the regression calculations. Another reason for using the probit transformation is that it linearizes the integrated normal signmoid curve. If all five test concentrations provide partial responses, then one can likely expect probit analysis to give reliable results when estimating the EC_{50} and its confidence interval provided the

fit is reasonable. Generally, probits are regressed on log-dose as opposed to dose.

The major drawback to using probit analysis in this situation is that the method does not make full use of the continuous aspect of the response variable. Probit analysis only requires that the response variable be quantal. Consequently, the approach literally wastes information by not using it.

The probit transformation can be applied to the response variable and then regressed, using least squares regression, on dose or log-dose. This approach has the same advantage as probit analysis in that it tends to linearize sigmoid-type curves. Therefore, it is appropriate to utilize data with response rates at or near 0 and 100 percent. In addition, this approach makes use of the fact that the response variable is a continuous measure. This approach, when the fit is reasonable, should give the most reliable EC_{50} estimate and possibly a narrower confidence interval than the other approaches.

Several other transformational approaches that might be tried (when the probit transformation regressed on dose or log-dose doesn't fit the data) are regress response on log-dose, response on the square root of dose or response on the inverse of dose.

If none of the above-mentioned linear regression transformations produce an acceptable linear function, then a non-linear sigmoid function such as: GROWTH = a/(1 + bc^{DOSE}) or GROWTH = 1/(a + $b^{-c*DOSE}$) could be fit to the data. The problem with using nonlinear functions is that in these cases three parameters, a, b, and c (rather than two

as in simple linear models), should be estimated. This generally requires more data for comparable fits. However, these functions will fit the data.

For each of the above methods--simple linear, probit analysis, linear regression via the various transformations, and non-linear regression--generalized lack-of-fit tests can be conducted to determine whether the chosen regression technique adequately describes the experimental data. Since there are twenty shell growth values for each treatment (test concentration), the appropriate statistical procedure (except in the case of probit analysis) is to conduct an "F Test for Linearity." The comparable test for probit analysis is the Chi-square goodness-of-fit test. If the computed F value for linearity is large, then the linear regression does not adequately describe the data and the EC_{50} value and confidence interval estimates are suspect. If the computed F value is acceptable (i.e., P less than or equal to .05 or P less than or equal to .10), them there is no reason to doubt that the data have been sufficiently described with the regression function and it would be appropriate to compute the EC_{50} and confidence intervals required by the test guideline.

c. Bioconcentration Test

The bioconcentration data (tissue test substance concentration) should be determined and recorded separately for each oyster, if possible, and of course also identifying the test chamber from which each oyster was taken. Certainly under the conditions necessitated by some chemical analyses where large amounts of tissue are needed for an analyses, this may not be possible. However, for a

bioconcentration test, duplicate samples are a necessity for establishing whether steady-state has been reached. It is not uncommon for bioconcentration data to vary half an order of magnitude from sample to sample. Therefore, there should be duplicate sample measurements for each sample period. Duplicate sample values are required for computing whether steady-state has been reached and for accurate computation of uptake and depuration rates, regardless of the statistical methods used. A minimum of four or more sample values for each sample period is recommended. The oysters sampled at each period from each test chamber should be individually analyzed. The control oysters can be pooled before analysis unless the chemical of interest or its metabolites are found or are expected in the control oyster samples, since the controls serve only to identify accidental and unknown contamination of test oysters from uncontrolled sources.

The variance of each sample period is likely to increase as the tissue concentrations increase, thus for statistical purposes multiple oyster samples at each sample period is necessary for determining when stead-state is reached for calculating a suitable 95 percent confidence interval.

An estimate of the time to steady state, the steady-state BCF, and the time to 95 percent elimination should be made for each compound tested. If steady-state has not been observed during the maximum 28 day exposure period or if 95 percent elimination has not been achieved during 14 days depuration, data generated during these tests should be used to estimate these values. The BIOFAC program developed by Blau and Agin (1978) uses nonlinear regression techniques to

estimate the uptake and depuration rate constant, the steady-state BCF, the time to reach 90 percent of steady-state, the time to reach 50 percent elimination and the variability associated with each estimate.

To date, there is no one specific method recommended for identifying the time to 95 percent elimination of accumulated residues. However, it is still of value to have it reported. The problems associated with calculating this 95 percent point are:

1) identifying the shape of the depuration curve as to whether it is linear or curvilinear;
2) if it is curvilinear, what curve best fits the data; and
3) are the data sufficiently good to allow extrapolation to estimate the 95 percent point?

Bioconcentration data is best displayed as log or natural log (ln) of the measured residue concentration on the vertical axis and time (linear) on the horizontal axis. The uptake curve will be exponential and increasing until leveling off at steady-state. This uptake curve is well represented by the standard kinetic uptake function

$$\text{Residue} = \text{Conc.} * K_1/K_2 * (1-e^{K2*t}).$$

This function has been shown to accurately represent most uptake data and has been used to determine uptake rates for oysters. However, there is no general function that consistently and adequately represents the depuration curve;

an appropriate choice should be made based on each data set. The common description of the observed problem is that chemicals partition within the oyster into different tissues (compartments) that depurate the chemical differentially, thus causing the depuration curve to be more complex and to vary for different data. It is important that the curve fits the data reasonably well since extrapolation is usually required to obtain the 95 percent depuration point. Generally, a non-linear parameter estimation statistical model can be used to describe the depuration data.

Since the same curve does not typically fit data of this type, a goodness-of-fit test should be conducted. If such a test were completed successfully, then extrapolation using the equation is more reasonable.

The final decision to be made is what 95 percent depuration level is to be reported? The reference value is the steady-state bioconcentration value; it is chosen as 100 percent uptake (0 percent depuration) and is normally reported in ug or mg per g or kg of tissue. At this point two options are available, 1) calculate 95 percent of the steady-state value in concentration units, or 2) calculate 95 percent of the steady-state value in log units. The more acceptable method is the latter. The following example will illustrate the difference in the methods.

> Assume steady-state oyster concentration equals 500 ug chemical per g oyster (500 ppm). Using the linear method for computing the 95 percent depuration endpoint: 95% X 500 ppm = 475 ppm; and 500 ppm - 475 ppm = 25 ppm is the endpoint. The time required for depuration to 25 ppm would be reported. Using the log

method for computing the 95 percent depuration endpoint: 95% X ln (500 ppm) = .95 X 6.21 = 5.90; and 6.21 - 5.90 = .31. Then the antilog of .31 = $e^{.31}$ = 1.36 ppm is the endpoint. The time required for depuration to 1.36 ppm would be reported. The last value, 1.36 ppm, is the actual 95 percent reduction (depuration) endpoint. This is because we are dealing with a ln-dose vs. time relationship and all computations and comparisons should be made on the ln transformed data with final back transformation to normal units for reporting. If 25 ppm were reported, the 25 ppm endpoint would represent only 48 percent depuration (.48 X 6.21 = 2.98; 6.21 - 2.98 = 3.23; and $e^{3.23}$ = 25 ppm).

In view of this example it is clear that the exact method of computing the 95 percent endpoint should be reported along withg the time required for depuration to this point.

In summary, the following procedures should be utilized for analyzing oyster bioconcentration data:

1) Accurately tabulate and quality assure the residue data, exposure concentrations, and sampling procedures and periods.
2) Compute the desired multi-compartment kinetic and/or non-linear parameter statistical equation using log residue and linear time data.
3) Plot the resulting curve(s) and data points.

4) Conduct a lack-of-fit test to determine whether the resulting equation(s) satisfactorily describe the data.

5) If satisfied that extrapolation is reasonable, compute the steady-state concentration, BCF, and 95 percent depuration endpoint using log transformed data. Back transform the endpoints to original concentration units for reporting.

The resulting constants (K_1, K_2, etc.) are required for constructing the computed curve and for estimating the time to 95 percent depuration.

d. Temperature Measurements

In order to substantiate that temperature was maintained within specified limits, it will be necessary to measure and record temperature throughout the test. Requisite instrumentation is readily available, easy to maintain, and should not increase complexity or costs of the test. Temperatures should be recorded hourly to prevent any severe fluctuations in temperature that might affect growth processes and/or chemical uptake.

B. Test Conditions

1. Test Species

a. Selection

The Eastern oyster, Crassostrea virginia (Gmelin), serves as a valuable indicator of biologically damaging pollutants in estuaries due to a number of important characteristics. First, the oyster is a long-lived sedentary filter feeder that is unable to move away from exposure to environmental contaminants nor close its shell for excessively long periods of time to avoid exposure. It

accumulates organic (both biological and chemical) and inorganic substances from aquatic ecosystems in a manner which accurately reflects environmental changes and quality (Galtsoff 1964).

Second, the oyster is economically important as a commercial and recreational fishery resource and as a human food source. For example, 1977 commercial landings were valued at $52 million (Council on Environmental Quality 1979).

Third, the oyster occurs naturally over a wide geographic range and is locally abundant from Maine to the Gulf of Mexico. This wide geographic range allows comparative studies of control and exposure organisms under differing environmental conditions.

Fourth, the oyster is readily cultured throughout its life cycle under controlled conditions (Maurer and Price 1967, Epifanio and Mootz 1976, Loosanoff and Davis, 1963). In a properly equipped and maintained facility, the oyster is a hardy species that can be maintained for long periods of time with minimal effort.

Fifth, numerous morphological, physiological and pathological studies have been completed on the oyster (Wilbur and Yonge 1964, Galtsoff 1964 and Sindermann 1970). It has been claimed that the oyster is the best known, most studied marine organism (Galtsoff 1964).

Sixth, Crassostrea virginica has been used extensively as a bioassay organism because of its known sensitivity to a wide variety of toxicants (LaRoche et al. 1973). It has been shown to be an effective bioconcentrator of aromatic hydrocarbons (Lee et al. 1978, Anderson 1978), the insec-

ticides kepone (Bahner et al. 1977, Hansen et al., 1976), DDT (Lowe et al. 1971, Butler 1967) and chlordane (Parrish et al. 1976), and various heavy metals (Frazier 1975, 1976, 1979 a,b). In addition, most specific responses of oysters to their environment have been studied and quantified including shell deposition rate, breeding temperatures, glycogen content, salinity requirements, numbers of reproductive cells, diseases and predators, and soft/hard tissue ratios.

Although no formal comparison of bioconcentration factors among bivalves has been published, Butler (1967) presented data which show that, in general, oysters bioconcentrate insecticides to a greater degree than most other common bivalves. Average five-day bioconcentration factors for seven pesticides ranged from 500 to 700 for the hard clam, marsh clam, and asiatic clam to 1200 for the oyster and 3000 for the soft shell clam. Butler concluded that, on the basis of its greater bioconcentration factors, the large body of knowledge concerning its biology, its sessile nature and its extensive range, the oyster makes an excellent biological monitor.

The voluminous literature on oyster biology is scattered through numerous scientific publications. However, the following references serve as suitable entries into the field: Galtsoff (1964), LaRoche et al. (1973), Sparks (1972), and Loosanoff and Davis (1963).

b. Sources

Oysters may be cultured in the laboratory, purchased from culture facilities, or collected from a natural population in an unpolluted area, free from epizootic

disease. Procedures for collecting, transporting and holding oysters are described in APHA, 1975. All oysters used for a particular test should be from the same source. Test oysters should not have been used in a previous test, either in a treatment or in a control.

c. Size

The test guidelines recommend using oysters between 30 and 50 mm in height. This range represents a synthesis of the wide range of sizes reported in the literature. Various workers have used oysters as small as 29 mm (Parrish et al. 1976) and as large as 120 mm (Scott and Middaugh 1978). Typically, however, experimental oysters have ranged from 40 to 60 mm.

Butler and Lowe (1978) and APHA (1975) recommend using small (25 to 50 mm) oysters because they are active over a wider range of temperatures and because they need less space. The ASTM (1980) recommends 40 to 60 mm. Therefore, in light of past experience and current recommendations, a size range of 30 to 50 mm is justified.

d. Condition

Oysters should be in a prespawn condition of gonadal development prior to and during the test. A test is unacceptable if oysters spawn during the test. For this reason, and in consideration of test temperature, it is recommended that oysters from natural areas be collected and tested in the spring of the year. Gonadal condition of oysters should be more certain in stocks obtained from culture facilities. Prespawn condition should be confirmed by measuring the condition and gonadal index of a randomly selected representative sample of oysters to be used for

testing by the method of Scott and Middaugh (1978) and by preparing and examining histological sections of tissues from the same oysters by the method of Tripp (1974) to determine gonadal condition and to additionally ensure the population is not diseased.

Although several authors utilized oysters from natural populations for bioassay tests (Schimmel et al. 1978, Rawls 1977), other investigators utilized laboratory-reared oysters (Conger et al. 1978, Cunningham and Tripp 1973). As demonstrated by Scott and Middaugh (1978), determining the physiological condition of oysters is very important in minimizing test variabilty. The depletion of gametes and glycogen that occurs during spawning would certainly make bioconcentration data impossible to analyze, and thus should be avoided by ensuring test oysters remain in a prespawn condition.

Oysters collected from a natural population should be collected at those times known to be free from influences of recent spawning, such as the spring of the year. Gamete production can be monitored by gross observation of individual oysters and semiquantitative measurements of gonad development can be made by the method of Tripp (1974). Oysters which are laboratory-reared should be examined prior to use to determine if their growth and shell thickness is consistent with known population averages (Pruder and Bolton 1978).

Oysters are susceptible to a number of pathogens that may result in epizootics and mass mortalities in both natural and cultural populations (Sindermann 1970). It is necessary to determine that purchased oysters do not

originate from epizootic disease areas.

In addition to taking the physiological condition of the oysters into account, examinations for parasitism and disease should be conducted to ensure to the investigators that the response of the oysters to the toxicant is not influenced by such factors. Common oyster diseases and procedures for their assessment are outlined in Cheng (1970), Couch et al. (1974), Galtsoff (1964), Sindermann (1970) and Sparks (1972).

Oysters with shells heavily infested with mudworms (Polydora websteri) should not be used. the mudworm forms black areas on the inner faces of oyster shells and make the shells brittle (MacKenzie and Shearer 1961). Heavily infested oysters may become weakened and eventually die (Roughley 1922, 1925). Oysters can be protected from mudworms to some extent if they are reared off the bottom (Loosanoff and Engle 1943).

2. Maintenance of Test Species

a. Feeding

The test guidelines permit supplemental feeding if natural plankton concentrations are too low to support oyster growth, or if artificial seawater systems are used. This statement, of course, leaves open the question of what plankton concentrations are adequate. This question cannot, at present, be answered with any degree of accuracy. Epifanio, et al. (1975) discussed the relationship between filtration rate and algal densities and concluded that the variability caused by temperature, animal size, particle composition, and density prevents an accurate specification of oyster nutrition. Their discussion presents information

suggesting that at 20°C oysters feed most efficiently at algal densities around 2 x 10^5 cells per ml. However, there are apparently no studies of the minimum necessary algal densities at various temperatures for various sizes of animal.

In the absence of adequate information on oyster nutrition in the wild, the best policy would appear to be test any source water suspected to be inadequate to supply growth. Juvenile oysters should be held in the testing system to determine rates of growth over an extended period of time. This will give an estimate of the system's ability to meet the demands of the flow-through bioassay.

It should be pointed out that most estuarine and nearshore waters will contain adequate quantities of phytoplankton during the period when water temperatures are suitable for testing. It is particularly true if the testing facility is located near an area which supports natural oyster populations. Therefore, it is unlikely that food availability will be a major factor when ambient water of suitable quality is used.

If supplemental feeding is necessary, the methods and materials employed by experimental aquaculture facilities should be utilized. Basically, these consist of culturing two or three algal species - Isochrysis galbana, Monochrysis lutheri and Thallasiosira pseudonna have been used successfully by the University of Delaware (Epifanio et al. 1975 Epifanio and Mootz 1976) - to be fed to the oysters either as the sole ration, or as a supplement to the natural algal flora. Although the actual algal culture presents no particular difficulties, the additional manpower and capital

costs of supplemental feeding make it an undesirable strategy. Ukeles (1971) has reviewed the nutritional requirements of shellfish.

3. Facilities

a. General

The requirements for facilities as set forth in the test guidelines are intended to ensure that the conditions in the test chambers are as uniform as possible and that the actual concentrations of test chemical in the test chambers are similar to the intended concentrations.

The test guidelines require that flowing seawater be utilized. Static test design cannot be utilized due to problems in maintaining the oysters in a state of good health. The flow-through system more closely simulates the natural exposure process, eliminating problems associated with accumulation of organic material (and associated bacteria which could lower dissolved oxygen) and toxic metabolic products. Test chemicals are more thoroughly mixed in a flow-through system and problems of sorption are reduced.

Galtsoff (1964) found that oysters held in flowing seawater at Woods Hole, Massachusetts, deposited a median of 1.4 milligrams of shell material per centimeter squared of shell surface per day during the growing season. With sufficient, suitable phytoplankton food in the dilution water, Epifanio et al. (1975) found that a small oyster between 30 and 50 millimeters in height may deposit as much as 1.0 millimeter of peripheral new shell per day. Most laboratory systems which have been designed to hold and study the toxic response of oysters have employed a minimum

flow-through volume of five liters of water per oyster per hour (Butler et al. 1960).

b. Construction Materials

Due to the toxicity of many heavy metals at low concentrations (USEPA 1976) and the ability of metal pipe, galvanized sheeting, laboratory equipment, etc., to leach metals into water, no metal other than stainless steel (preferably #316) should be used. In the same manner, unaged plasticized plastic (PVC) should not be used due to the high toxicity of a main component, di-2-ethyl-hexyl phthalate (Mayer and Sanders 1973) and the ability of DEHP to leach into aquaria systems from these materials (Carmignani and Bennett 1976). To avoid any possible stress due to exposure to low levels of metals, phthalates, and other potential contaminants, #316 stainless steel, glass and perfluorocarbon plastics should be used whenever possible and economically feasible. If other materials should be used, conditioning to a continuous flow of heated dilution water should be performed for a minimum of 48 hours.

c. Test Substance Delivery System

To maximize the accuracy and precision of test results developed through the use of this test guideline, the quantity of test chemical introduced by the test chemical delivery system should be as constant as possible from one addition of test chemical to the next. Fluctuations in the quantity of test chemical introduced into the test chamber may result in abnormally high or low response value (e.g. EC50's) of the test organisms and in a wider spread of response values in replicate tests. The greater the

variation in the quantity of test chemical introduced, the greater the potential for abnormalities and spread of the response values.

Variations in the quantity of dilution water entering the test chambers during a given time interval may also create undesirable differences in test conditions between test chambers. The concentrations of dissolved oxygen and test chemical in a test chamber, for example, may decrease more rapidly in chambers having lower flow rates. Differences between test chambers in the concentration of dissolved oxygen, test chemical, metabolic products and degradation products, individually or in combination, may result in response values for the test organisms which are inaccurate.

The following criteria presented by Hodson (1979) should be considered when selecting or designing a toxicant delivery system: 1) if the delivery of dilution water stops, so should delivery of the toxicant 2) consistency in delivery amounts throughout the test periop 3) independence from electrical failure 4) independence from temperature and humidity fluctuations 5) capacity to deliver small quantities 6) ease of construction, with few moving parts and 7) ease of operation.

Any one of several toxicant devices can be used as long as it has been shown to be accurate and reliable throughout the testing period. The greater the variation in the quantity of test chemical introduced, the greater the spread of response values measured during testing. Syringe injector systems (Barrows et al. 1980, Spehar et al. 1979), metering pump systems (Veith et al. 1979), and modified

proportional dilutors (Macek et al. 1975, Neeley et al. 1974) have been reported to be successfully used.

The solubility of the test compound should also be taken into account in selecting an appropriate delivery system. If the compound can be solubilized in water, a device capable of delivering amounts of test solution greater than 1 milliliter (ml) will probably be needed. If a carrier is required, a system capable of accurately delivering small amounts, less than 100 microliters (ul), will probably be required to minimize the carrier concen-tration in the test solution.

Each system should be calibrated prior to starting the test to verify that the correct proportion of test chemical to dilution water is delivered to the appropriate test chambers.

d. Test Chambers and Loading

Flexibility is allowed in the design of test chambers as long as adequate space is provided for test oysters to meet loading requirements. As a guideline use the USEPA Bioassay Procedures for the Ocean Disposal Permit Program Manual (USEPA 1978), which recommends glass or fiberglassed wood containers measuring 64 x 38 x 10 cm deep (25 x 14x4 inches) to provide adequate space for 20 oysters. Such containers permit adequate circulation of the water, while avoiding physical agitation of the oysters by the water current. These containers hold about 18 L at 75% capacity and at a flow rate of 100 L $hour^{-1}$, will provide 5 L of water $hour^{-1}$ $oyster^{-1}$. Small oysters were reported to feed and grow readily under these conditions.

Silicone adhesive is the preferred bonding agent for

constructing test chambers. It is inert, and the solvent it generally contains (acetic acid) is easily washed away or volatilized from the system. A minimum amount of the adhesive should contact the test solution because it may absorb test materials. If large amounts of the adhesive are needed for strength, it should be applied to the outsides of chambers and apparatus to minimize contact.

e. Flow-through System

The test guidelines require that flowing seawater be utilized. Static test design cannot be utilized due to problems in maintaining the oysters in a state of good health. The flow-through system more closely simulates the natural exposure process, eliminating problems associated with accumulation of organic material (and associated bacteria which could lower dissolved oxygen) and toxic metabolic products. Test chemicals are more thoroughly mixed in a flow-through system and problems of sorption are reduced.

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f. Cleaning

Before use, test systems should be cleaned to remove dust, dirt, and other debris and any residues that may remain from previous use of the system. Any of these substances may affect the results of a test by sorption of test materials or by exerting an adverse effect on test organisms. New chambers should be cleaned to remove any dirt or chemical residues remaining from manufacture or accumulated during storage. Detergent is used to remove hydrophobic or lipid-like substances. Acetone is used for the same purpose and to remove any detergent residues. It is important to use pesticide-free acetone to prevent the contamination of the chambers with pesticides which influence the outcome of the test. Nitric acid is used to clean metal residues from the system. A final thorough rinse with water washes away the nitric acid residues. At the end of a test, test systems should be washed in preparation for the next test. It is easier to clean the equipment before chemical residues and organic matter become embedded or absorbed into the equipment.

g. Dilution Water

The test guidelines require the availability of an adequate and dependable supply of clean, unfiltered estuarine or ocean water. This water should not deviate substantially from the desired temperature and salinity ranges (APHA 1975, Pruder and Bolton 1978). General requirements for a water supply and water system are described in Standard Methods (APHA 1975) and in Epifanio and Mootz (1976). If necessary, artificial seawater may be used for limited studies (Conger et al. 1978). However, the

large volumes required to maintain the flow and loading plus the need to add food to maintain growth makes use of artificial seawater problematic. Refer to Spotte (1979) for methods to prepare and mix large volumes of artificial seawater.

The flow-through system should supply at least one liter of water per oyster per hour. The ASTM Proposed Standard Practice (ASTM 1980b) recommends one liter per hour, although this preliminary recommendation has been questioned in the review process, and is likely to be changed.

Behind the ASTM recommendation is a factor which has only recently received the attention it merits, namely the problem of waste disposal. Dilution water containing a potentially hazardous test chemical cannot be discharged directly into natural waters. Some form of preliminary treatment is necessary. As the amount of dilution water increases, treatment facilities and costs go up. These considerations dictate the use of the lowest flo rate which will support oyster growth.

The recommendation that the flow rate be at least one liter per oyster per hour is clearly at the lower end of the range of reported values. Parrish et al. (1976) exposed oysters to chlordane in 7.5 liters of water per oyster per hour. Scott et al. (1979) used five liters per hour. Schimmel et al. (1978) supplied approximately six liters per oyster per hour to test sodium pentachloro-phenate, but 11 liters to test lindane and BHC (Schimmel et al. 1977). Scott and Middaugh (1978) used at least five liters per oyster per hour in their acclimation tanks. Standard Methods (APHA 1975) advises holding spawning stock in at

least seven liters per animal per hour, but those are larger oysters (7.5 to 15 cm) than are used in the test guidelines. For test oysters, "a minimum of five liters per hour of seawater per oyster provides adequate growing conditions" (APHA 1975). However, recent experience at the USEPA Gulf Breeze Laboratory (Schimmel, personal communication) and at private facilities (Parrish, personal communication) has demonstrated the effectiveness of using one liter. Since that flow rate is economical and has been proven effective, its use in the test guidelines is justified. The one liter figure is a minimum; if a facility can support a higher flow rate, it should by all means use the higher flow. It is important that the flow of water be constant. If the flow is interrupted, the oysters will quickly deplete the food supply in the stagnated water and will increase the levels of metabolic wastes. These factors will cause the oysters to close their shells, which will invalidate the tests.

Many previous tests utilizing the oyster as a test organism were conducted at salinity regimes native to the testing facility. Thus, tests conducted by Schimmel et al. (1978) were conducted at a salinity range between 18 and 23 parts per thousand, whereas those completed by Cunningham (1976) were run at salinities close to full-strength seawater (34 parts per thousand).

Salinity is difficult to control in a natural flow-through system, and controlling it introduces an element of artificiality to the test. Therefore, the dilution water should be drawn from an area where the expected range of salinity is within the oyster's optimum.

Regardless of the system used, salinity should be monitored daily during the test. If significant changes occur, due to severe weather changes or system malfuncion, for example, the validity of the test is challenged and the test should be repeated.

To avoid possible inconsistencies and inaccuracies in test results, healthy oysters are needed for use in toxicity tests. There is also a need to determine that the dilution water, whatever its source, is able to maintain the oysters to be used in a healthy condition for the duration of the holding and testing periods.

An appropriate way to make that determination is to place oysters in the dilution water for an extended period of time and observe their behavior, growth and development. Ideally, those observations should be made by an experienced oyster biologist familiar with certain stress reactions which are difficult for an untrained observer to identify.

Particulate matter and gas bubbles, if present in the dilution water, may clog the toxicant delivery system used in flow-through tests. Gas bubbles also may cause excessive loss of volatile test chemicals. Either circumstance may alter the concentration of test chemical to which the test oysters are exposed. To avoid this problem an apparatus capable of removing particulate matter or gas bubbles from the dilution water may be required. If the dilution water is heated prior to use, it may also be necessary to de-saturate the water from >100% of oxygen saturation. Penrose and Squires (1976) describe a suitable apparatus for this.

An adequate supply of dissolved oxygen should be available to the oysters. To facilitate this, the dilution water or holding water should be at 90-100% of oxygen saturation prior to delivery to the holding tanks or test system.

h. Carriers

Carriers may be used to aid in the dissolution of test compounds into dilution water only after significant efforts to dissolve it in dilution water or dilution water stocks have failed. Schoor (1975) believes that the use of a carrier may interfere with the uptake of the test compound by the test organism; if the carrier molecules affect the adsorption of the test compound at the gill surface, a change in the rate of transport into the test organism may result. The author also states that the use of a carrier may increase the concentration of compound in the test solution above solubility by creating a stable water emulsion.

Since there is little information available on the effects of carriers on oysters, follow precautionary usage procedures that have been established with fish. When a carrier is used, triethylene glycol (TEG), dimethyl formamide (DMF), or acetone may be used. The solvents should be tried in the order stated due to their relative toxicity to fathead minnows as reported by Cardwell et al. (manuscript 1980). The minimum amount should be used and the concentration of TEG should not exceed 80 mg/l, the MATC (maximum acceptable toxicant concentration) value. Concentrations of DMF and acetone should not exceed 5.0 mg/l, the MATC for DMF. Although there is no MATC value for

acetone, its acute toxicity is similar to that of DMF.

Ethanol should not be used due to its tendency to stimulate the excessive growth of bacteria in the test chambers.

4. Environmental Conditions

a. Dissolved Oxygen (See Section on Dilution Water)

b. Temperature

It is desirable to standardize the range of test temperatures to the extent possible so as to avoid variability between different laboratories in widespread geographic areas. Also, tests on some specific substances will vary significantly at temperatures as much as 10°C apart, a range commonly experienced between, for instance, New York and South Carolina. Waldichuk (1974) presented data showing such a phenomenon in the case of cadmium. Gunter (1957) set forth the relationship between oyster growth and temperature, as determined by regional location. Based on Gunter's data and work conducted by Butler (1953), it is clear that regional temperature differences should be taken into account in establishing experimental bioassay conditions. Most bioassay tests utilizing the oyster were conducted at ambient temperatures of the dilution water at the testing facility. Tests conducted by Schimmel et al. (1978) were conducted at ambient temperatures between 7 and 9°C; those completed by Cunningham (1976) were run at times at temperatures as high as 25°C.

Within its natural range, Crassostrea virginica is found at great temperature extremes, ranging from 1°C to 46°C

(Epifanio et al. 1976). However, the optimal temperature of oyster growth has been reported as between 15°C and 25°C in the Gulf Coast (Collier 1954) and between 13°C and 22°C in Long Island Sound (Loosanoff and Nomeiko 1949). Although oysters in the southern range, such as in the Gulf of Mexico, have a greater growth per year, he maximum growth per day occurs during the summer in oysters located in the mid-Atlantic region. Loosanoff (1958) found that oysters from Long Island have maximum ciliary (feeding) activity at approximately 25°C. Cunningham (1976) and others observed declining shell growth during cold ambient water temperature periods. Laboratory studies conducted by Epifanio and Mootz (1976) utilized a controlled range between 16° and 26°C throughout the year. The ASTM (1980b) proposed standards for tests with oysters call for a test temperature between 3°C and 28°C. Butler and Lowe (1978) recommend that the source water be between 15°C and 30°C. The test guidelines specify 20°C (with a permissable short term deviation within 15°-25°C) because it maximizes filtration, growth, and is sufficiently low that oysters can be maintained in a prespawn condition. In addition, it will tend to diminish the influence of disease on test results because the fungus, Dermocystidium marina, causes high levels of mortality above 25°C (Hewatt and Andrews 1957).

According to Stickney (1979), C. viginica requires a temperature range of 21 to 27°C. for spawning and can be conditioned to spawn within about 6 weeks in the winter if exposed to a temperature range of 23 to 24°C., even though spawning naturally occurs in the spring.

The 20°C test temperature was selected as a compromise,

striking a balance between the temperature that would maximize the physiological activities of the oyster (i.e. ciliary movement, water transport, metabolism, etc.) which would thereby enhance the uptake and exposure to the test solution on the one hand, and on the other hand assuring that the oysters would not spawn on the other hand. The ideal physiological maximum temperature for the oyster is approximately 25°C, but oysters spawn above 20°C. Since the oyster approaches maximum physiological activities at 20°C, and since this temperature does not induce oysters to spawn, it was selected. It is realized that temperature variation may occur in controlling the volumes of water required in flow-through systems. Since prolonged exposure of oysters to temperatures above 20°C may induce spawning, it is preferred that variations in test temperature be held to a minimum or held to temperatures below 20°C. The effect of temperature on the oyster is discussed in Galtsoff (1964).

A standardized temperature is ordinarily desirable in bioassay testing. This is because the toxicity of many substances varies with temperature (Tucker and Leitzke 1979, Frazier 1979a, Waldichuk 1974).

The effects of sudden temperature changes on organisms may range from death to temporary impairment of physiological functions, depending on the acclimation temperature, the magnitude of the temperature change, the temperature tolerance of the species, and the circumstances and duration of the exposure. To avoid any undue stress, accurate temperature control devices should be used to both maintain constant temperatures, and to gradually increase or decrease the temperature during acclimation procedures.

Such mechanisms have been described by Defoe (1977) and Lemke and Dawson (1979).

c. Light

The duration and intensity of light are not important environmental variables in oyster tests. Oysters have no visual organ and are not sensitive to light (Galtsoff, 1964). Therefore, no lighting regime is required.

d. Salinity (See Section on Dilution Water)

C. Reporting

The sponsor should submit to the Agency all data developed during the test that are suggestive or predictive of oyster toxicity and bioconcentration. In addition, information on water quality, experimental design, equipment, and oyster condition are required beause these data have a bearing on the validity of the test. If testing specifications are followed, the sponsor should report that specified procedures were followed and present the results. If alternative procedures were used instead of those recommended in the test guideline, then the protocol used should be fully described and justified.

Test temperature, chemical concentrations, test data, concentration response curves, and statistical analyses should all be reported. The justification for this body of information is contained in this support document. If species other than the recommended were used, the rationale for the selection of the other species should be provided.

III. Economic Aspects

The USEPA awarded a contract to Enviro Control, Inc. (1980) to provide the Agency with an estimate of the cost for performing the oyster acute toxicity and

bioconcentration tests according to the test guidelines. Two estimates were provided; a protocol estimate and a laboratory price survey estimate.

The protocol best estimate for the oyster acute toxicity test was $1480. This estimate was prepared by separating the guideline into individual tasks and estimating the hours to accomplish each task. Hourly rates were then applied to yield a total direct labor charge. An overhead rate of 115 percent, other direct costs (for laboratory supplies and reagents) of $75.00, a general and administrative rate of 10 percent, and a fee of 20 percent were then added to the direct labor charge to yield the final estimate.

Enviro Control estimated that differences in salaries, equipment, overhead costs and other factors between laboratories would result in as much as 50 percent variation from this estimate. Consequently they estimated that test costs could range from $740 to $2221.

The laboratory price survey best estimate was $900.00 for the oyster acute toxicity test. Two laboratories supplied estimates of their costs to perform the tests according to this guideline. These costs ranged from $700.00 to $1100.00. The reported estimate is the mean value calculated from the individual costs.

The protocol best estimate for the oyster bioconcentration test was $7680. This estimate was prepared by the same method of the oyster acute toxicity test, with the exception that the other direct costs totaled $250. The test cost was estimated to range from $3840 to $11,520.

The laboratory price survey best estimate was $8092 for the oyster bioconcentration test. Four laboratories

supplied estimates ranging from $4,000 to $10,000. The reported estimate is the mean value calculated from the individual costs.

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PENAEID SHRIMP ACUTE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

PENAEID SHRIMP ACUTE TOXICITY TEST

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

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

(1) "Death" means the lack of reaction of a test organism to gentle prodding.

(2) "Flow-through" means a continuous passage of test solution or dilution water through a test chamber, holding or acclimation tank with no recycling.

(3) "LC50" means that experimentally derived concentration

of test substance that is calculated to have killed 50 percent of a test population during continuous exposure over a specified period of time.

(4) "Loading" means the ratio of test organism biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber.

(c) Test procedures--(1) Summary of the test. Prior to testing, the bottoms of the test chambers are covered with 2-3 cm of sand and then filled with appropriate volumes of dilution water. The flow is adjusted to the rate desired to achieve loading requirements. Penaeid shrimp are introduced into the test chambers according to the experimental design. The shrimp are maintained in the test chambers for a period of 3-7 days prior to the beginning of the test. The test begins when the test substance is introduced into the test chambers. The rate of flow is adjusted to maintain the desired test substance concentration in each chamber. The shrimp are observed during the test; dead shrimp are counted, removed, and the findings recorded. Dissolved oxygen concentration, pH, temperature, salinity, test substance concentration and other water quality characteristics are measured at specified intervals in selected test chambers. Data collected during the test are used to develop concentration-response curves and LC50 values for the

test substance.

(2) [Reserved]

(3) Range-finding test. (i) A range-finding test should be conducted to determine the test substance concentrations to be used for the definitive test.

(ii) The shrimp should be exposed to a series of widely spaced concentrations of test substance (e.g. 1, 10, 100 mg/l, etc.).

(iii) A minimum of five penaeids should be exposed to each concentration of test substance for up to 96 hours. No replicates are required and nominal concentrations of the chemical are acceptable.

(4) Definitive test. (i) The purpose of the definitive test is to determine the concentration-response curves and the 48- and 96- hour LC50 values with the minimum amount of testing beyond the range-finding test.

(ii) A minimum of 20 shrimp per concentration should be exposed to five or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32 and 64 mg/l). An equal number of shrimp should be placed in two or more replicates. If solvents, solubilizing agents or emulsifiers have to be used, they should be commonly used carriers and should not possess a synergistic or antagonistic effect on the toxicity of the test substance. The

concentration of solvent should not exceed 0.1 ml/l. The concentration ranges should be selected to determine the requested concentration-response curves and LC50 values. The concentration of test substance in test solutions should be analyzed for chemical concentration prior to use and at designated times.

(iii) Every test should include controls consisting of the same dilution water, conditions, procedures and shrimp from the same population or culture container, except that none of the chemical is added.

(iv) The dissolved oxygen concentration, temperature, salinity and pH should be measured at the beginning of the test and at 24, 48, 72 and 96 hours in each test chamber.

(v) The test duration is 96 hours. The test is unacceptable if more than 10 percent of the control organisms die or appear to be stressed or diseased during the 96 hour test period. Each test chamber should be checked for dead shrimp at 3, 6, 12, 24, 48, 72 and 96 hours after the beginning of the test. Concentration-response curves and 48- and 96- hour LC50 values should be determined along with their 95 percent confidence limits.

(vi) In addition to death, any abnormal behavior or appearance should also be reported.

(vii) Distribution of shrimp among test chambers should be randomized. In addition, test chambers within the testing area should be positioned in a random manner or in a way in which appropriate statistical analyses can be used to determine the variation due to placement.

(viii) The concentration of dissolved test substance (that which passes through a 0.45 micron filter) in the test chambers should be measured as often as is feasible during the test. The concentration of test substance should be measured:

(A) in each chamber at the beginning of the test and at 48 and 96 hours after the start of the test;

(B) in at least one chamber containing the next to the lowest test substance concentration at least once every 24 hours during the test;

(C) in at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system. Among replicate test chambers of a treatment concentration, the measured concentration of the test substance should not vary more than 20 percent.

(5) [Reserved]

(6) Analytical measurements (i) Test chemical. Deionized water should be used in making stock solutions of the test substance. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test substance in a sample should be validated before beginning the test by appropriate laboratory practices. An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) Numerical The number of dead shrimp should be counted during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves. A 48- and 96- hour LC50 and corresponding 95 percent intervals should be calculated.

(d) Test conditions--(1) Test species--(i) Selection. This test should be conducted using one of three species of penaeid shrimp: Penaeus aztecus (brown shrimp), Penaeus duorarum (pink shrimp), or Penaeus setiferus (white shrimp). Post-larval juvenile shrimp should be utilized. Shrimp may be reared from eggs in the laboratory or obtained directly as juveniles or adults. Shrimp used in a particular test should be

of similar age and be of normal size and appearance. Shrimp should not be used for a test if they exhibit abnormal behavior or if they have been used in a previous test, either in a treatment or in a control group.

(ii) Acclimation. During acclimation, shrimp should be maintained in facilities with background colors and light intensities similar to those of the testing areas. In addition, any change in the temperature and chemistry of the dilution water used for holding and acclimating the test organisms to those of the test should be gradual. Within a 24 hour period, changes in water temperature should not exceed 1°C, while salinity changes should not exceed 2 °/oo.

(iii) Care and handling. Upon arrival at the test facility, the shrimp should be transferred to water closely matching the temperature and salinity of the transporting medium. Shrimp should be held in glass tanks of 30 liter capacity or larger. No more than 22 to 24 shrimp should be placed in a 30 liter tank unless the flow-through apparatus can maintain dissolved oxygen levels above 60 percent of saturation. With species of the genus Penaeus, a minimum flow rate of 7.5 l/g body weight day should be provided. Larger flows, up to 22 l/g body weight day, may be desirable to insure dissolved oxygen concentrations above 60 percent of saturation and the removal of metabolic products. The

period of acclimation to ambient laboratory conditions should be at least 4-7 days.

(iv) Feeding. Penaeid shrimp should not be fed during testing. Every two or three days during the acclimation period, shrimp should be fed fish pieces approximately 1 cm^2. Uneaten food should be removed daily.

(2) Facilities--(i) Apparatus. (A) Facilities which may be needed to perform this test include: flow-through tanks for holding and acclimating penaeid shrimp; a mechanism for controlling and maintaining the water temperature and salinity during the holding period; apparatus for straining particulate matter, removing air bubbles, or aerating water when necessitated by water quality requirements; and an apparatus providing a 12-hour light and 12-hour dark photoperiod with a 15-to-30 minute transition period. Facilities should be well ventilated, free of fumes and free of all other disturbances that may affect test organisms.

(B) two to three centimeters of acid-washed sand, free of excess organic matter, should be placed in the bottom of test chambers.

(C) Test chambers should be loosely covered to reduce the loss of test solution or dilution water due to evaporation, mimimize entry of dust and other particles and prevent escape of the shrimp.

(ii) Cleaning. Test substance delivery systems and test chambers should be cleaned before each test following standard laboratory practices.

(iii) Construction materials. Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect test results.

(iv) Dilution water. (A) Natural or artificial seawater is acceptable as dilution water if shrimp will survive in it without signs of stress, such as unusual behavior or discoloration. Shrimp should be acclimated and tested in dilution water from the same origin.

(B) Natural seawater should be filtered through a five micrometer filter with a pore size < 20 microns prior to use in a test.

(C) Artificial seawater can be prepared by adding commercially available formulations or by adding specific amounts of reagent-grade chemicals to deionized water. Deionized water with a conductivity less than 1 u ohm/cm at 12°C is acceptable for making artificial seawater. When deionized water is prepared from a ground or surface water source, conductivity and total organic carbon (or chemical oxygen demand) should be measured on each batch.

(v) Test substance delivery system. Proportional diluters, metering pumps or other suitable systems should be used to deliver test substance to the test chambers. The system used should be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test substance in each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow through a test chamber should be equal to at least five times the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent among test chambers or across time.

(3) Test parameters. Environmental parameters of the water contained in test chambers should be as specified below:

(i) Temperature of 23 ± 1°C.

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation. Aeration, if needed to achieve this level, should be done before the addition of the test substance. All treatment and control chambers should be given the same aeration treatment.

(iii) The number of shrimp placed in a test solution should not be so great as to affect results of the test. Loading requirements will vary depending on the flow rate of dilution water. The loading should not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 12 hours light and 12 hours darkness, with a 15-30 minute transition period.

(v) Salinity of 20 ± 3 Percent.

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

(1) The nature of the test, laboratory, name of the investigator, test substance and dates of test should be supplied.

(2) A detailed description of the test substances should be provided. This information should include the source, lot number, composition, physical and chemical properties and any carrier or additives used.

(3) Detailed information about the shrimp should be provided: common and scientific names, source of supply, age, history, weight, acclimation procedure and feeding history should be reported.

(4) A description of the experimental design including the number of test solution concentrations, number of replicates and

number of shrimp per replicate should be provided.

(5) The source of the dilution water, its chemical characteristics (e.g., salinity) and a description of any pretreatment.

(6) A description of the test chambers, the depth and volume of solution in the chamber, the number of organisms per treatment, the number of replicates, the loading, the lighting, the test substance delivery system and flow rate expressed as volume additions per 24 hours.

(7) The concentration of the test substance in each test chamber before the start of the test and at the end.

(8) The number of dead shrimp and measurements of water temperature, salinity, and dissolved oxygen concentration in each test chamber should be recorded at the designated times.

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

(10) Recorded data for the holding and acclimation periods (temperature, salinity, etc.).

(11) Concentration-response curves should be fitted to mortality data collected at 24, 48, 72 and 96 hours. A statistical test of goodness-of-fit should be performed.

(12) For each set of mortality data, the 48- and 96- hour

LC50 and 95 percent confidence limits should be calculated on the basis of the average measured concentration of the test substance. When data permits, LC50 values with 95 percent confidence limits should be computed for 24 and 72 hour observations.

(13) The methods used in calculating the concentration-response curves and the LC50 values should be fully described.

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August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

PENAEID SHRIMP ACUTE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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Technical Support Document for Penaeid Acute Toxicity Test

I. Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-7 which uses Penaeid shrimp to evaluate the toxicity of chemical substances. The Document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures and conditions prescribed in the Test Guideline. Technical issues and practical considerations relevant to the Test Guideline are discussed. In addition, estimates of the cost of conducting the test are provided.

II. Scientific Aspects

A. Test Procedures

1. General

A flow-through bioassay technique was chosen because of several distinct advantages over static exposure methods. Continuously flowing seawater not only simulates the natural exposure process but, when used as a laboratory tool, eliminates problems associated with the accumulation of organic matter and toxic metabolic products. Flow-through techniques should be used with materials which have a high oxygen demand, are highly volatile, are unstable in aqueous solution, are readily biodegradable, or are removed from test solutions in significant amounts by the test organisms. Toxicants flowing through this system are more thoroughly mixed and loss due to sorption to sediments and feces is minimized. Flow-through techniques for holding and acclimation of shrimp provides a smooth transition to actual testing.

For acute tests, 96 hours is a convenient interval of time for starting and completing a test in a normal five-day work week, and is better than shorter periods for estimating cumulative and other chronic effects. Because set-up is the most expensive portion of a test, a 96-hour test is only slightly more expensive than 24 or 48 hour tests. Yet additional data on the LC50's over time and the observation of other abnormal effects that do not appear in shorter tests are gained for this slight increase in cost. Although the 48 hour test can reduce costs, the 96-hour toxicity test was selected for the penaeid test guidelines because of greater probability for determining the incipient LC50 (threshold limit for acute toxicity) through extension of the toxicity curve.

2. Range-Finding Test

The concentration range for the definitive tests is normally chosen based on the results of a range-finding test. Range-finding tests with penaeid shrimp are usually short-term (24-96 hour) flow-through bioassays which utilize fewer organisms per test substance concentration than required for the definitive test. In all cases, the range-finding test is conducted to reduce the expense involved without having to repeat a definitive test due to inappropriate test substance concentrations.

3. Definitive Test

The concentration range for the definitive test is chosen based on results on the range-finding test. By using a minimum of five test substance concentrations, partial kills both above and below the median 50 percent mortality level are probable and will help define the concentration-

response curve. The more partial kills, the better the definition of the concentration-response curve. The slope and shape of the concentration-response curve can allow insight into the mode of action of a chemical and will allow estimation of the effects of lower concentrations upon the test organisms. In addition, by having partial kill data, a greater array of statistical methods can be used to determine an LC50 value.

A sample size of 20 shrimp permits several combinations of replicates and sample sizes to be used. The use of replicate samples allows an analysis of variance to be performed on the results.

Measurements of test substance concentrations at designated periods during the flow-through test allows documentation of real test concentrations at appropriate periods under acute conditions.

Chemical and physical parameters (temperature, pH, dissolved oxygen, and salinity) are recorded at specified times to permit evaluation of the biological conditions present for shrimp survival in test water.

Specified observations on mortality characteristics are designed to allow an adequate evaluation of dose-response effects in acute penaeid tests. In addition, these defined observation times allow greater comparability of dose-response data between different chemicals and laboratories.

B. Test Conditions

1. Test Species

a. Selection

The prime considerations in the selections of test organisms for toxicity tests are: (a) their sensitivity to a

wide spectrum of test substances; (b) their geographical distribution and abundance; (c) their recreational, economic and ecological importance; (d) their availability as test organisms, and existence of established culture. Penaeid shrimp have become the most valuable marine species harvested from U.S. Coastal waters by commercial fisherman (Temple, 1973). In 1972, an estimated 190.6 million pounds of shrimp were harvested from coastal waters; 87 percent of this harvest was from the Gulf. During 1974, the Louisiana brown shrimp catch alone was 27.4 million pounds, and was valued at 18 million dollars (Temple, 1973; Knudsen et al., 1976).

Perhaps the most important quality of penaeid shrimp for toxicity testing is their consistently high sensitivity to test substances. In virtually all comparative toxicological studies in the laboratory, penaeid shrimp proved the most sensitive marine organism to a variety of toxins. Pink shrimp have been used repeatedly in the last 10 years (Lowe 1971, Tagatz 1975; Schimmel 1979; Parrish 1976; Nimmo and Bahner 1976); white shrimp and brown shrimp have also been used successfully in toxicological tests (Nimmo and Bahner 1974; Curtis 1979).

Penaeid shrimp are also sensitive at sublethal doses of toxins; this allows the maximum amount of information to be gleened from each test. For example, pink shrimp were one of a selected group of estuarine animals used to assess the effects of mirex leaching into the environment; toxicity was latent and became more apparent with increasing length of exposure. Under stress they showed darker coloration, loss of equilibrium and a cessation of burrowing behavior,

measures which provided valuable sub-lethal effects data (Tagatz et al. 1975).

In juvenile pink shrimp exposed to Mirex, Lowe (1971) observed the first case of delayed toxicity. Mortality at the end of seven days was 25 percent, but increased to 100 percent by day 11 even after the shrimp were removed to mirex-free water. In a flow-through acute toxicity test, Schimmel, et al. (1979) found pink shrimp especially sensitive to the insecticides EPN and leptophos; in this case there was 20 percent mortality at non-detectable (nominal) concentrations of these insecticides in test water.

An additional point to consider is the suitability of the species for cultivation, since cultured shrimp are preferable for use in toxicity testing. Previous history of an organism is a major variable affecting the potential response to a test substance. While the tropical species P. monodon and P. orientalis have been shown to grow most rapidly under high-density cultivation, these species are not representative of those organisms residing in U.S. coastal waters. Penaeus aztecus (brown shrimp) and P. setiferus (white shrimp) grew significantly longer in low densities ($25m^2$) than high densities ($166m^2$). However, of the 9 shrimp species studied, brown shrimp had the second highest survival rate at high densities (Forster and Beard 1974). In the same study, penaeid shrimp were shown to be less variable in individual growth rates than Machrobracium spp., the freshwater prawn.

Some limitations in the use of penaeid shrimp have been reported. High mortalities due to cannibalism, cramped test

conditions, or control mortality can be expected in culture (Curtis et al. 1979; Tagatz et al. 1976).

In view of the continued successful use of penaeid shrimp for toxicity testing in many laboratories, and their sensitive and varied response to sublethal toxic concentrations, they are the species of choice for this flow-through bioassay. In fact, because of their suitability as a test organism and their value as an economic resource, a wealth of literature is available for reference in developing culture and testing techniques, as well as a comparative toxicology data base.

b. Sources

Whether collecting organisms for testing or for culturing purposes, a great deal of care should be taken to avoid stress and insure survival in transport to the laboratory. Shrimp should be collected from unpolluted sources and measurements of water temperature, salinity and pH should be taken at capture time. This allows for successful acclimation to laboratory conditions. Taking organisms from areas of known high levels of parasitism, disease, pollution, or where deformed individuals are found should be avoided to insure valid results.

The following salient points are emphasized by APHA (1975) when collecting organisms for bioassay purposes; in general, great care should be taken to insure the shrimp are not damaged in the collection, transfer and transporting process:

(1) When seining or using trawls, make short hauls; keep gravel, sand and other debris out of net.

(2) Do not expose delicate, easily damaged stages to the air; juvenile shrimp can be transferred with dip nets.

(3) Do not collect too many animals at one time.

(4) Do not crowd the organisms during transportation and watch for signs of stress; observe animals in the laboratory for additional signs of stress.

Juvenile and adult shrimp are most easily collected by hand-held seines or boat trawls. For test organisms, one should select shrimp of uniform size and in the post-larval stages. Do not mix stages within the test. For culture purposes, the preferred method is to collect gravid females and allow them to spawn in the laboratory. It may not yet be feasible to breed penaeid shrimp prawns in captivity (Walker 1975). As a guide for distinguishing life stages in penaeids, Rose et al. (1975) has suggested these total length criteria: juvenile (25 mm); subadult (90 mm); and adult (140 mm). Shrimp have been success-fully transported by motor vehicle in plastic bags or buckets filled with oxygenated seawater (Mock 1974). It is recommended that test organisms come from a controlled environment such as a laboratory maintenance system. This insures that shrimp are uniform in age, size and experi-mental history.

2. Maintenance of Test Species

a. Handling and Acclimation

Tanks for holding and acclimation should be identical to those used for testing, which eliminates further stressing of shrimp by an additional transfer. Water should be of the same temperature and salinity as water from the collection site; a gradual change in water quality parameters should be

made to acclimate the shrimp to the test conditions. This gradual period of acclimation should be at least 7 days; and up to 2 weeks has been suggested (APHA 1975). It has been shown that activity responses to tidal rhythms do not fade until 7 days of captivity have passed. It is important that shrimp be similar in their activity cycles before testing begins (Subrahmanyam 1976).

Following the initial holding period, shrimp should be randomly assigned to their respective test chambers and held there until testing begins, again eliminating further handling. However, as will often be the case, extra shrimp will need to be maintained in separate tanks. No more than 22 to 24 shrimp should be kept in a 30 liter tank with a flow-through mechanism to allow maintenance of dissolved oxygen (DO) levels above 60 percent of saturation. Flow rates should be great enough to remove metabolic products and food build-up which have been demonstrated to cause high mortality (Mock 1974). A minimum flow of 7 l/g day shrimp should be maintained, while flows up to 22 l/g day may be needed. Holding tanks should attain preliminary test condition within 7 days; gradually acclimate shrimp to salinity and temperature conditions required by the test so as to minimize stress.

Inspection for parasitism and disease should be made during the acclimation period; diseased shrimp should never be used in tests. Methods for detecting and treating the following prawn diseases are given in Delves-Broughton (1976) and should be referreed to when needed; shell disease, black module, vibriosis, hasplosporidian infection, filamentous gill growth, filamentous bacteria on eggs, and

systematic fungus disease. Spontaneous muscle necrosis is the result of abrupt changes in temperature and salinity; treatment is discussed by Lakshmi (1978). When antibiotic use is necessary, oxytetracycline and oleandonycin are suggested. They are 99 percent bactericidal at high doses and do not significantly lepress respiration in shrimp (Chan and Lawrence, 1974). If antibiotics are used in the water of test chambers (during acclimation), they should be removed before testing begins. This is possible when the chelator EDTA is substituted at a concentration of 10 mg/l of seawater (APHA 1975).

b. Feeding

During holding and acclimation period juvenile or adult penaeids may be fed cut-up fish. Fillet from mullet, grouper, or other abundant species should be cut into pieces about 1 cm^2 and fed, one per shrimp, every 2 or 4 days. Uneaten food should be removed every 24 hours to reduce fouling. Protozoal stages of shrimp are generally fed algae, chiefly the diatoms Thalassiosira and Skeletonema (Cook & Murphy, 1969, Cook 1967, Mock 1974). Techniques for culturing the diatom, and mechanisms for maintaining them in shrimp rearing tanks are discussed in detail by Mock (1974). Equipment and procedures for the continuous mass culture of algae as a food source are also found in APHA (1975). Add algae as a concentrate either fresh (centrifuges) or frozen. Do not add algal culture medium to acclimation or test water since it is toxic to shrimp (Mock 1974). The number of algal cells necessary to rear a population of larval shrimp during the protozoal stages are as follows (APHA 1975):

Protozoel I	Skeletonema	50,000 cells/ml
Protozoel II	Skeletonema	150,000 cells/ml
Protozoel III	Tetraselmis	20,000 cells/ml

It was found that the addition of several algal foods insure higher rates than additions of a single algal species at comparable concentrations (Mock 1974). When only one species is used for larval shrimp, it should be Skeletonema costatum.

Brine shrimp (Artemia sp.) nauplii have been used extensively as food for the mysi stage through the fourth post-larval stage. In tests of food preference in brown and white shrimp, both species demonstrated a preference for nauplii of brine shrimp (Artemia) when given a choice of diets. Brown shrimp were more flexible, but still preferred Artemia. Karim and Aldrich (1976) tested various commercial foods and brown shrimp preferred Vio Bio Fish Flour and white shrimp preferred Silvray Fish Feed. It was stressed that these prepared foods not be recommended for general use until their effect on survival and growth are demonstrated to be favorable.

Therefore, Artemia should be used for post-larval stages. The quantities required are:

Mysis	Artemia nauplii	3/ml
Mysis	Artemia nauplii	3/ml
Mysis	Artemia nauplii	3/ml
Post-larval I-IV	Artemia nauplii	3/ml

A recent study by Johns and Walton (1979) reported that adult Mysidopsis bahia fed Artemia spp. from San Pablo Bay, California exhibited increased mortality, did not reproduce and showed reduced growth rates. In contrast, both juvenile and adult mysid shrimp fed Artemia spp. strains collected from Brazil, Australia, Italy and Utah maintained high survival and growth rates. These results imply that nutritional quality of Artemia, possibly associated with pesticide or heavy metal contamination, can significnatly influence test results and, therefore, should be considered.

There are several basic methods of crustacean aquaculture: extensive culture (using large outdoor enclosure); intensive culture (small outdoor tanks); and indoor intensive (high-density flow through tanks in the laboratory). The indoor intensive system is most practical for use with bioassay techniques because of the relative ease of controlling the aquaculture environment. Tanks are stocked at high densities (for this guideline, not more than 22-24 adults per 30 liter tank). In order to prevent fouling of the system, it is necessary to circulate water through the tank to maintain high DO levels. The overflow test water may overflow to a drain or be recycled through a biological filter (Walker 1975).

A temperature range of 28°-30°C and a salinity range of 27-35°/oo are most satisfactory for shrimp larval culture. Since the shrimp will be later used in acute toxicity tests, they should be acclimated to the presceibed test conditions by post-larval stages. The specific requirements of each species should be considered.

Penaeid shrimp can be reared regularly from the egg to post-larval stage in the laboratory. Cook and Murphy (1969) have described, in detail, equipment and techniques for conditioning, spawning and rearing large numbers of shrimp larvae from eggs. Methods of rearing shrimp larvae for experimental studies have been described by Cook (1967). These references should be followed closely.

3. Facilities

a. General

The delivery of constant concentrations of test substances is required to reduce variability in test results. Large fluctuations in test substance concentration will give abnormally high or low responses, depending upon the mechanism of toxic action. Proportional diluters and metering pumps (Mount and Brungs 1967) have been found to provide constant concentrations and are widely used.

Proportional diluters operate on a sequential filling and emptying of water chambers. The water chambers are cali-brated to contain a measured amount of water. Separate water chambers can be provided for toxicant and diluent waters. Diluent and toxicant waters are mixed in siphon tubes and delivered to the replicate test chambers. The cyclic action of the diluent is regulated by a solenoid valve connected to the inflow dilution. The system is subject to electrical power failure, so an alternate emergency power source is recommended.

The proportional diluter is probably the best for routine use. It is accurate over extended periods of time, nearly trouble free, and has fail-safe provisions (Lemke et al. 1978). A small chamber to promote mixing of toxicant-

bearing water and dilution water should be used between the diluter and the test chamber for each concentration. Since replicate chambers are used in this test, separate delivery tubes can be run from the mixing chamber to each replicate test chamber.

Calibration of the toxicant delivery system should be checked carefully before and after each test. This should include determining the flow rate and toxicant concentration through each test chamber. The general operation of the system should be checked daily.

Alterations in the design of the proportional diluter, such as the use of six or more concentrations have been useful in some situations (Benoit and Puglisi 1973).

b. Construction Materials

In an excellent review of potential sources for chemical contamination in the culture system and laboratory, Bernhard and Zattera (1970), stress the importance of avoiding chemical contamination in culturing marine organisms. Therefore, choice of laboratory equipment on toxicant testing is critical.

Several materials such as rubber and polyvinyl chlorides have been found highly toxic; and should never be used in culture or testing of marine organisms. Teflon (algoflon), Perspex, Polyethylene, Tygon, Polypropylene, Polycarbonates (Makrolor) and Polyester (Gabraster) have been shown to be non-toxic and suitable for experiments with marine organisms.

All pipes, tanks, holding chambers, mixing chambers, metering devices, and test chambers should be made of materials that minimize the release of chemical contaminants

into the dilution water or the adsorption of the test substances. Chemicals that leach from construc-tion materials can stress test organisms, or possibly act synergistically or antagonistically with test substances to give inaccurate results. Generally, undesirable substances are not leached from perfluorocarbon plastic, titanium, and borosilicate glass; in addition, the tendency of these materials to adsorb substances, is minimal. Rubber, copper, brass, galvanized metal, lead and epoxy resins should not come in contact with dilution water, stock solution, or test solutions because of the toxic substances they contain (USEPA 1975). All containers and pipe need to be conditioned before use in order to leach and wash away any undesirable residues that may be present.

c. Test Substance Delivery System

Flow-through systems should have the capability to vary and maintain water temperature, dissolved oxygen, and salinity at desired levels during holding, acclimation and testing. Penaeid shrimp are extremely sensitive to fluctuations in these parameters, which affect test validity. Tagatz et al. (1975) reported that a slight (3-4°C) change in water temperature resulted in significant increases in the mortality rates of juvenile Peneaus duorarum exposed to mirex. These mortality increases were greater than those due to longer (3x) exposure times (Lowe et al. 1971). Similarly, salinity decreases have been shown to cause significant increase in mortalities of P. aztecus exposed to Aroclor (Nimmo and Bahner 1975). Combined, or synergistic effects of dissolved oxygen (DO), temperature and salinity on the toxicity of toxaphene on pink shrimp

were identified and are discussed in detail by Courtenay and Roberts (1973).

Shrimp health and survival are directly affected by water quality and handling. Physically stressed organisms are not valid test subjects. Attention to husbandry and routine water quality monitoring are of paramount importance in prevention of disease (Delves-Broughton and Poupard 1976). Spontaneous muscle necrosis (exhibited as white foci on the 4th, 5th and 6th abdominal segments) in brown shrimp (P. aztecus) was induced in healthy shrimp by over-crowding, lowering dissolved oxygen (DO) levels, or changing physico-chemical conditions (Lakshmi et al. 1978). High mortality in adult brown shrimp from gas bubble disease was caused by supersaturation of dissolved oxygen (DO) in water (Supplee and Lightner 1976). This occurred with dissolved oxygen (DO) levels exceeding 250 percent saturation. Morbidity and mortality not only hinder the progress of testing, but alter those toxic effects of concern, thus invalid ting tests. Salinity and temperature also affect burrowing behavior, metabolic rate, and cause increased aggression; such aberrations cause distorted test results. For example, hyperactivity, was shown in brown shrimp within 30 minutes of a change from optimum conditions (Lakshmi et al. 1978).

The dilution water should be filtered through a twenty micrometer filter (or smaller) to sufficiently reduce the amount of suspended sediments, organic material and biological organisms (phytoplankton, zooplankton, fungi, bacteria, etc). This will minimize the confounding of results associated with the differential sorption of the test substance on cell walls, clay particles, etc. which in

turn may enhance or reduce the availability of the test substance to the shrimp.

Accumulation of gases can cause adverse effects; therefore, a device for removing air bubbles may be necessary (Penrose and Squires 1976). When the dissolved oxygen (DO) in the dilution water is less than 60 percent, aeration is suggested. Culturing techniques recommend 70-100 percent saturation for penaeid shrimp (Forster and Beard 1974; Supplee and Lightner 1976). A device for simulating natural photoperiod with transitions from light to dark is suggested so that conditions can be optimized for shrimp (Drummond and Dawson 1970).

d. Test Chambers

Choice of test chamber size should consider both the needs of the test organism and the requirements of the test. Chamber size should reflect the appropriate loading require-ment using the number of organisms specified in the experimental design. P. aztecus and P. setiferus showed a significant difference in length attained when grown in low ($25/m^2$) and high $166/m^2$) densities (Forster and Beard 1974). Stress caused by crowding has been shown to induce latent viral infections in healthy pink shrimp (Couch 1974).

Penaeid shrimp are large organisms. Juvenile individuals range from 0.4 mm up to approximately 25 mm in length (Rose 1975). In the 60x30x30 cm high container recommended, 20-22 juvenile shrimp may be housed, as long as the total live weight is no more than 50 g per chamber. A substrate of two to three centimeters of organic free sand, permits the shrimp to burrow. Screens for chamber tops are recommended (APHA, 1975) to prevent the escape of shrimp from the test chambers.

e. Cleaning of Test System

Before use, test systems are cleaned to remove dust, dirt, other debris, and residues that may remain from the previous use of the system. New chambers should be cleaned to remove any chemical or dirt residues remaining from manufacture or accumulated during storage and construction. Detergent is used to remove hydrophobic or lipid-like substances. Acetone is used for the same purpose and to remove any detergent residues. It is important to use pesticide-free acetone to prevent the contamination of the chambers with pesticides. Nitric acid can be used to clean metal residues from the system.

At the end of a test, test systems should be washed in preparation for the next test or storage. This will prevent chemical residues and organic matter from becoming embedded or absorbed into the equipment.

Priming the system with dilution water before use allows equilibrium to be reached between the chemicals in the water and the materials of the testing system. The testing system may sorb or react with substances in the dilution water. Allowing this equilibrium to be established before exposure of the test shrimp to the test substance lessens the chances of water chemistry changes during a test.

f. Dilution Water

A constant supply of dilution water is required to maintain consistent experimental conditions. An interruption in flow or changes in water quality parameters can change the chemistry of the test system and possibly affect the response of the test population. Therefore, the results of a test with variable dilution water quality are not comparable to tests run under constant conditions and

the results are more difficult to interpret.

For acute toxicity tests, a minimum criterion for an acceptable dilution water is that healthy test organisms will survive for the duration of the acclimation period without showing signs of stress. Signs of stress in penaeid shrimp include darkened coloration, cessation of burrowing behavior, loss of equilibrium, and antennae-chewing (Tagatz 1976; Forster and Beard 1974). Investi-gators should be familiar with normal shrimp behavior patterns, as well as gross physical changes which may occur during testing.

Since shrimp have both estuarine and marine phases during their life cycle, the salinity of dilution water is of prime importance. Determination of the desired salinity was made by considering the natural habitat characteristics, laboratory results, and individual species preferences. The most important test requirement will be to maintain a constant salinity level for the entire holding and testing period. It is important also to monitor dissolved oxygen (DO) levels; they should be kept above 60 percnt of saturation. The pH of the test solution appears to be less important to the health of shrimp. Some studies have suggested a pH of 8.3 to 8.7 for white shrimp (Curtis et al. 1979) and 8.0 for euryhaline species in general (Kester et al. 1967; Zaroogian et al. 1969).

Natural seawater, obtained from a point source with similar characteristics to those designated for the test species, or water from an area where the test organisms were obtained, is preferrable to artificial sea water. Dilution water should be of constant quality and should be uncontaminated. Contaminants may affect the results directly and indirectly. For example, low levels of

organochlorine chemicals have been shown to increase the prevalence of latent viral infections in pink shrimp (Couch, 1975). This is as important during holding as during testing.

If alternatives to reconstituted seawater are used, they should meet the following specifications for contaminant levels (USEPA 1975).

Suspended Solids	< 20 mg/l
TOC	< 10 mg/l
Un-ionized ammonia	< 20 ug/l
Residual Chlorine	< 3 ug/l
Total organophosphorus pesticides	< 40 ng/l
Total organochlorine pesticides plus PCB's	< 50 ng/l

Maintaining the desired salinity level in natural waters often poses a problem. When possible, obtain water from an area of high salinity and obtain low salinities by adding either deionized or glass distilled water of a satisfactory quality. To increase salinity, use a strong, natural brine, which can be obtained by freezing and then partially thawing seawater. This procedure can be used if limited amounts of seawater are needed. However, it is recommended that artificial seawater be used when large quantities of dilution water are needed (APHA, 1975).

g. Controls

Controls are required for every test to assure that any effects which are observed are due to the test substance and not to other factors. These may include effects from construction materials, environmental factors, vapors, stressed test organisms, etc.

Ten percent mortality may be anticipated due to inherent biological factors. In a test chamber of 20 organisms, this amounts to two deaths. Any increase above this may be attributed to conditions of the test. The ten percent mortality figure is representative of a wide variety of organisms including both fish and invertebrates captured from the wild. Capture tends to stress organisms so there is more likelihood of stress related death. In addition, invertebrates are generally more vulnerable to handling injury. If penaeid shrimp are raised under controlled conditions, they are generally more healthy than are captured organisms, therefore, fewer should die during a test because of inherent biological factors.

h. Carriers

Carriers can effect test organisms and can possibly alter the form of the test substance in water. Therefore, it is preferable to avoid the use of carriers in toxicity tests unless required to dissolve the test substance. Since carriers can stress or adversely effect test organisms, the amount of carrier should be kept to a minimum. A recommended maximum is 0.1 ml/L (APHA 1975).

Triethylene glycol and dimethylformamide have been shown to exert the least influence on test organisms and test substances of several carriers that have been used in testing marine organisms. Acetone and ethanol have a stronger tendency to reduce the surface tension of the water and therefore decrease oxygen saturation (Veith and Comstock 1975; Krugel et al., 1978; APHA 1975).

i. Randomization

The positions of test chambers are randomized to prevent conscious or unconscious biases from being introduced.

These biases can be in environmental conditions such as temperature and lighting, shrimp selection and distribution, diluter system function, etc.

4. Environmental Conditions

a. Dissolved Oxygen

Large variations in flow rates to chambers will result in environmental differences between chambers. Parameters such as dissolved oxygen (DO) and test substance concentration can decrease more rapidly in chambers with low salinities. High salinities can be decreased by adding either deionized or glass distilled water of a satisfactory quality. To increase salinity, use a strong, natural brine, which can be obtained by freezing and then partially thawing seawater. This procedure can be used if limited amounts of seawater are needed. However, it is recommended that artificial seawater be used when large quantities of dilution water are needed (APHA, 1975).

b. Light

The three species of penaeid shrimp have been shown to have a nocturnal peak in activity when held in captive laboratory conditions. Burrowing frequencies and durations were highest during bright light hours, and shrimp were more active above the substrate during dark hours. Of the three species tested, P. setiferus (white shrimp) was least influenced by the light schedules and was more active than either pink or brown shrimp, being exposed on the substrate day and night (Wickham and Minkler, 1975). This is supported by catch data for shrimp which show higher catch levels in daylight for white shrimp than the other species.

Cool white fluorescent lighting should be used. White light has been determined necessary for maintaining a circadian burrowing pattern. A 15-30 minute transition period between light and dark cycles is suggested. It appears that shrimp initiate activity rhythm changes during this transition period (Bishop and Herrnkind 1976). Thus, the 12:12 light-dark schedule using white light not only mimics environmental conditions, but also allows for equalizing the time organisms spent exposed to test substances in the water and substrate. Furthermore this standardization facilitates comparison between tests using different species.

c. Temperature and Salinity

Penaeid shrimp occur naturally in estuarine waters where temperature and salinity vary over a wider range than in oceanic waters. A review of the literature of toxicity testing demonstrates the broad range of conditions over which penaeid shrimp have been maintained.

Brown shrimp (Penaeus aztecus) occur in waters ranging from 15°-35°C in temperature and 9-40 °/oo in salinity (Copeland and Bechtel, 1974). In culture, growth was found to be optimum between 15-20°C (Temple, 1973).

Pink shrimp Peneaus duorarum) occur naturally in waters where temperature and salinity vary from 5-38°C and 20-35°/oo respectively (Copeland and Bechtel, 1974). Optimal temperatures for growth are in excess of 20°C (Copeland and Bechtel, 1974).

White shrimp (Penaeus setiferus) are found in water ranging from 10-40°C and 0-38 °/oo in salinity (Copeland and Bechtel, 1974). Optimum temperatures for growth have been

shown to be between 15 and 20°C (Temple, 1973).

Thus there is considerable overlap in temperature and salinity requirements of the three penaeid species. Therefore, it is reasonable to select a single temperature and salinity level for testing purposes; tests should be conducted a a temperature of 23 ± 1°C and a salinity level of 20 ± 2 °/oo to minimize the difficulty in obtain-ing a suitable source of dilution water.

Furthermore, minimizing variability in testing conditions by specifying temperature and salinity conditions allows greater comparability of inter-laboratory test results and for the development of a comparative toxicology data base. An acceptable method for maintaining desired temperature and salinity ranges in flow-through bioassays with marine organisms is described in Bahner and Nimmo (1975).

C. Reporting

A coherent theory of the dose-response relationship was introduced by Bliss (1935), and is widely accepted today. This theory is based on four assumptions:

(1) Response is a positive function of dosage, i.e., it is expected that increasing exposure should produce increasing responses.

(2) Randomly selected animals are normally distributed with respect to their response to a toxicant.

(3) Due to homeostasis, response magnitudes are proportional to the logarithm of the dosage, i.e., it takes geometrically increasing dosages (stresses) to produce arithmetically increasing responses in test animal populations.

(4) In the case of direct dosage of animals, their resistance to effects is proportional to body mass. Stated another way, the treatment needed to produce a given response is proportional to the size of the animals treated.

The concentration-response curve, where percent mortality is plotted as a function of the logarithm of test solution concentration, can be interpreted as a cumulative distribution of tolerance within the test population (Hewlett and Plackett 1979). Experiments designed to measure tolerance directly (Bliss 1944) have shown that in most cases tolerance is lognormally distributed within an experimental population in most cases. Departures from the lognormal pattern of distribution are generally associated with mixtures of very susceptible and very resistant individuals within a population (Hewlett and Plackett 1979). In addition, mixtures of toxicants can produce tolerance curves which deviate significantly from the lognormal pattern (Finney 1971).

If tolerances are lognormally distributed within the experimental population, the resulting concentration-response curve will be sigmoidal in shape, resembling a logistic population curve (Hewlett and Plackett 1979). While estimates for the mean lethal dose can be made directly from the dose response curves, a linear transformation often is possible, using probit (Bliss 1934; Finney 1971) or logit (Hewlett and Plackett 1979) transformations.

Once the mortality data have been transformed, a straight line can be fitted to the data points. This line is more often fitted by eye (APHA 1975), but a least square

linear regression procedure is strongly recommended (Steel and Torrie 1960). From the regression equation, confidence limits can be determined for predicted mortality values. An additional advantage is that the significance of the slope of the regression line can be determined (Draper and Smith 1966). By using replicate experimental chambers, an analysis of variance can also be performed to determine whether deviations of data points from the regression line are random fluctuations and indicate whether a linear model is an appropriate representation of the data points (Draper and Smith 1976).

While values for the mean lethal dose, LC50, can be estimated graphically from the linearized concentration-response curve (APHA 1975), other techniques are preferable since the graphical method does not permit the calculation of confidence limits.

The probit method (Finney 1971) uses the probit transformation and the maximum likelihood curve fitting technique. The Litchfield and Wilcoxon method (1949) is a modified probit method which does not require partial kills, as does the unmodified probit method. The logit method (Ashton 1972) utilizes either the maximum likeli-hood or the minimum chi-square method (Berkson 1949) to estimate LC50. The moving average (Thompson 1947) is simple to apply but depends on the symmetry of the tolerance distributions to provide accurate estimates.

The moving average method can only be utilized to calculate the LC50. An additional disadvantage of this method is that confidence limits for LC50 cannot be calculated if partial kills are not available.

The lack of partial kills seriously impairs the utility of the probit, logit, and moving average methods. In situations where there are no partial kills, the binomial test (Siegal 1956) can be used to estimate the confidence limits around the LC50 value (Stephan, 1977). The LC50 value can be calculated from the relation:

$$LC50 = [(A) \times (B)]^{1/2}$$

where

A = concentration at which no organisms die
B = concentration where all organisms die

A and B are the confidence limits of the estimate and are significant above the 95 percent level since more than six test organisms are exposed at each concentration level (Stephan 1977).

If dose-response data is plotted for each 24 hour interval throughout the test, the LC50 determined from each curve can be plotted as a function of time, yielding an acute toxicity curve (APHA 1975). This curve approaches the time axis asymptotically, indicating the final or threshold value for LC50. The absence of a threshold LC50 may indicate the need for an acute test of longer duration.

III. Economic Aspects

The agency awarded a contract to Enviro Control, Inc. to provide us with an estimate of the cost for performing a flow-through acute toxicity test. Enviro Control supplied us with two estimates; a protocol estimate and a laboratory survey estimate.

Protocol Estimate

	range	mean
Acute	$510-$1529	$1019

This estimate was prepared by separating the guidelines into individual tasks and estimating the hours used to accomplish each task. Hourly rates were then applied to yield a total direct labor charge. An overhead rate of 115 percent, other direct costs of $105, a general and administrative rate of 10 percent and a fee of 20 percent were then added to the direct labor charge to yield the final estimate.

Laboratory Survey Estimate

	range	mean
Acute	$1000-$1450	$1234

The laboratory survey estimates were based on two laboratory estimates.

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ALGAL ACUTE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
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ALGAL ACUTE TOXICITY TEST

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

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

(1) "Algicidal" means having the property of killing algae.

(2) "Algistatic" means having the property of inhibiting algal growth.

(3) "ECx" means the experimentally derived chemical concentration that is calculated to effect X percent of the test

criterion.

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

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

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

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

(2) [Reserved]

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

(A) definitive testing is necessary

(B) test chemical concentrations for the definitive test.

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

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

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

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

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

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

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

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

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

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

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

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

algicidal effects by the following two methods:

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

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

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

(5) [Reserved]

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

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

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

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

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

test, either in a treatment or a control.

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

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

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

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

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

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

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

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

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

(3) Test parameters. (A) The test temperature should be maintained at 24°±1°C for Selenastrum and 20°±1°C for Skeletonema. Temperature should be recorded hourly during the test.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

ES-5
August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

ALGAL ACUTE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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TECHNICAL SUPPORT DOCUMENT FOR ALGAL ACUTE TOXICITY TEST

Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-8 which uses freshwater and marine algae to evaluate the acute toxicity of chemical substances. The Document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures and conditions prescribed in the Test Guideline. Technical issues and practical considerations relevant to the Test Guideline are discussed. In addition, estimates of the cost of conducting the tests are provided.

II. Scientific Aspects

A. Test Procedures

1. General. A balanced growth of algae in the aquatic environment is essential, but extremes in productivity may be detrimental to other organisms. Some algae are able to inhibit or stimulate the growth of other algae, for example Selenastrum can inhibit Microcystis growth in eutrophic water (Toerien et al. 1974). Inhibition of algal growth would alter the food web and reduce the productivity of ecosystems. The toxic effect of a chemical or other inhibitor may increase the susceptibility of algae to other environmental stresses (Fisher and Wurster 1973). Stimulation of algal growth may cause an algal bloom which may have negative aesthetic effects; may adversely affect commercial sport fisheries (Lightner 1978, Lovell 1979) and recreation; may impart unpleasant taste to drinking water; may release substances deleterious to aquatic animals,

and/or may indirectly kill aquatic organisms by creating anoxic conditions (Shilo 1964, Schwimmer and Schwimmer 1967). Stimulation of algal growth, while primarily a problem in eutrophic freshwaters, has created serious ecological problems in the open ocean as well. In the spring of 1976 and extending into the fall, there was an extensive algal bloom, dominated by Ceratium tripos, located off the New Jersey coast. The bloom, together with a dearth of storm activity, anomalous surface wind conditions, and unusually warm sea surface temperatures resulted in a huge anoxic area, 100 miles long and 40 miles wide which had a severe impact on the finfish and shellfish populations in the area. The immediate effects on commercial and sport fishes, lobsters, and shellfish were not entirely known. However, an estimated 59,000 metric tons of surf clams were killed (representing twice the annual U.S. harvest), and up to 50% of other shellfish populations sampled were killed. One commercial trawler reported up to 75% of fish collected were dead. It was predicted that these mortalities would affect recruitment, population size and harvests for years to come (Sharp 1976).

Another more commonly known phenomenon is the adverse effect caused by stimulated growth of toxigenic marine algae. Frequently explosive mass development of these organisms in the form of blooms and tides occur, resulting in fish kills, contaminated shellfish, and outbreaks of paralytic shellfish poisonings in humans. (Shilo 1964, Taylor and Seliger 1979).

Even when toxigenic organisms are not present in sufficient concentrations to affect human health, red tides

may reduce the market for shellfish because of adverse publicity (Council on Environmental Quality, 1979). Furthermore, the high concentrations of phytoplankton that occur during blooms can be harmful to shellfish because the rate of water transport by molluscs is reduced and feeding ceases (Galtsoff 1964).

Algal growth was selected to measure phytotoxicity for the following reasons:

- The selection of phytoplanktonic algae for toxicity testing is based upon their importance in aquatic ecosystems. Algae were one of the first cellular life forms, dating as far back as 3.1 billion years in the fossil record (Bold and Wynne 1978) and are numerous today. Because phytoplankton are ubiquitous, it is usually the case that most marine and freshwater ecosystems are based upon the primary production of phytoplankton (Stern and Stickle 1978). Primary production is of prime significance to estuarine energetics since the primary producers are at the base of the food web. In estuaries phytoplankton are the main primary producers in the water (Vernberg 1977).
- Algae convert inorganic carbon to organic carbon and liberate oxygen during photosynthesis. Thus, they are primary producers of food and energy for the lower trophic-level herbivores which in turn provide food for the upper trophic-level carnivores, generally fishes (Vance and Maki 1976). Some species fix nitrogen, required for the growth of vascular plants. Therefore, much of the

food people eat and the oxygen they breathe are the result of algal productivity.

- Inferences may be drawn from laboratory tests for inhibition or stimulation of algal growth as to the extent to which a chemical substance can interfere with primary productivity and nutrient cycling in lakes, streams, estuaries, and oceans. Further inferences may be drawn from algal bioconcentration data as to the potential of a chemical substance to bioaccumulate in food chains. However, in the natural environment there are too many factors acting to regulate algal populations which cannot be simulated in a simple laboratory test. The real value of the test guideline is to determine threshold toxicity values and to evaluate the relative toxicity of test substances to one another under rigidly controlled conditions.
- Algal testing has been well established in the literature. In 1967, the EPA began developing algal assays for evaluating the ecological effects of pollution to the environment. Initially designed for considering problems associated with eutrophication (Maloney and Miller 1975), algal assays have also been used to define the toxic effects of heavy metals (Davies 1978), pesticides (Schauberger and Wildman 1977, Walsh and Alexander 1980), oil spills (Corner 1978, Fisher and Wurster 1973, O'Brien and Dixon 1976, Vandermeulen and Ahern 1976), chemical substances (USEPA 1978 a,b,c, Harding and Phillips 1978), dyes (Little and

Chillingworth 1976), complex industrial wastes (USEPA 1978d, Walsh and Alexander 1980, Walsh et al. 1980) and natural organic components of fresh and marine water (Prakash and Rashid 1968). Over the years, extensive use of this test has sufficiently refined it to qualify as a standard method to measure water quality. Algal assays are recommended for use by the APHA (1975) USEPA (1977, 1978 a,b,c,d) and are currently under review by the American Society for Testing and Materials. Further discussion on the validity of applying algal assays in water quality assessment is found in Fitzgerald (1975); Joint Industry/Government Task Force on Eutrophication (1969); Leischman et al (1979); USEPA (1978b) Miller et al. (1978); Murray et al. (1971); Reynolds et al. (1974); and USEPA (1971, 1975a).

- The algal growth method is 1) relatively rapid, 2) inexpensive, 3) capable of being performed by persons with minimal technical training and 4) reproducible, using large numbers of organisms with sufficient replication and precision.

The test procedure involves assessment of algal growth in test chambers relative to controls by requiring a quantitative determination of algal cell numbers, and by recommending a) a qualitative appraisal of algal numbers and size by means of microscopic observation, and b) a determination of viability of growth-inhibited algae by means of mortal staining coupled with microscopic observation and/or subculturing. The test procedure is

simple because it requires only the combination of set amounts of test substance, nutrient medium and algae, and then monitoring the growth response 96 hours later. At the end of 96 hours a further assessment of growth and viability is recommended.

In the test the following procedures are required:

- Algal growth should be logarithmic at the beginning of the test and algal number should be determined.
- The number of algae should be determined at the end of the test.
- The concentration of chemical in the test solution should be determined at the beginning and end of the test and the concentration of chemical associated with the algal cells should also be determined.
- growth and bioconcentration data should be subjected to statistical analyses.

These requirements will ensure consistency and will minimize variability of the test results. The test also recommends testing of algicidal and/or algistatic chemical effects.

2. Range-Finding Test

It is recommended that a range-finding test be conducted prior to the definitive test in those instances where no information is available or can be elucidated on the phototoxicity of the test chemical. This approach should minimize the possibility that an inappropriate concentration series will be utilized in the definitive test and under certain circumstances may even preclude the need to conduct the definitive test. In order to minimize the cost and time

required to obtain the requisite data nominal concentrations are permitted, test duration may be shortened, replicates are not required and other test procedures and conditions are relaxed.

If test results indicate that the chemical is non-toxic or very toxic to algae and if definitive testing is not conducted, it is necessary to ascertain that the control algae have attained a logarithmic growth rate by 96 hours and that the test was conducted at the specified incubation temperature. These verifications establish that the algae tested were viable and that the test was properly conducted.

In some situations there may be enough information available on toxicity to select the appropriate concentration without a range-finding test. The range-finding test (or other available information) needs to be accurate enough to ensure that dose levels in the definitive test are spaced to result in concentrations above and below the EC-10 and EC-50 values for algal growth and mortality. If the chemical has no measurable effect at the saturation concentration (at least 1000 mg/l), it is considered relatively nontoxic to algal growth and definitive testing for effects on these processes is deemed unnecessary. In all cases, the range-finding test is conducted to reduce the expense involved with having to repeat a definitive test because of inappropriate test chemical concentrations.

3. Definitive Test

The specific requirements of the definitive test are the analytical determinations of chemical concentrations, the unbiased selection of algae for each treatment, the use of controls, the assessment of test validity, and the

recording, analysis, and presentation of data. These requirements assure that the chemical concentration - algae response relationship is accurately known, that chemical effects are not confounded by differential algal growth and that the relationships are clearly present. Reporting the occurrence of such abnormal effects as irregular cell size or shape, clumping, loss of chlorophyll, cell mortality, or other unusual effects provides qualitative data that further assist the assessment of phytotoxicity.

The purpose of the definitive test is to determine the EC-10, EC-50 and concentration-response curves for algal growth for each species tested with a minimum of testing beyond the range-finding test. The concentration range for the definitive test is based upon the results of the range-finding for that species. It is probable that each of the species tested may have a different estimated EC-50 based on the range-finding test and that more than five concentrations of a test substance in a geometric series may be needed to properly describe the dose-response relationship for either species being tested. By testing a minimum of five concentrations in a series per species the dose-response relationship will be better defined. The slope and shape of the dose-response curve can give an indication of the mode of action of the chemical and will allow estimatiion of the effects of lower concentrations on the algae.

The primary observations - number of algae per chemical and determination of the actual chemical concentrations employed in the definitive test, are needed to accurately describe the dose-response curve from which the EC-10 and EC-50 are calculated.

The recommended experimental design is the randomized complete block. As discussed by Hammer and Urquhart (1979), it is essential that the investigator randomly assign test containers to treatments to assure that each aliquot of algae has the same chance of receiving any of the treatments (exposure level of test chemical). To account for variation within the growth chamber and to increase the sensitivity for detecting treatment differences, small square blocks should be delineated in the growth chamber with randomization of treatment within blocks. Replication should occur over growth chambers (of the same type) as, in many cases, a within-growth chamber estimate of residual variance badly underestimates the between chamber estimate (Hammer and Urquhart 1979). This means that differences between growth chambers are often greater than differences between growth and environmental conditions within chambers.

4. Analytical Measurements

The actual chemical concentration used in the definitive test should be determined with the best available analytical precision. Analysis of stock solutions and test solutions just prior to use will minimize problems with storage (e.g., formation of degradation products, adsorption, transformation, etc.). Nominal concentrations are adequate for the purposes of the range-finding test. If definitive testing is not required because the chemical elicits an insufficient response at the 1000 mg/l level in the range-finding test, the concentration of chemical in the test solution should be determined to confirm the actual exposure level.

The pH of the test solution should be measured prior to testing to determine if it lies outside of the species optimal range. While it is recognized that algae may grow over a broad range of hydrogen-ion concentrations and typically exhibit a pH optima for logarithmic growth, this test guideline does not include pH adjustment for the following reasons: the use of acid or base may chemically alter the test substance making it more or less toxic, the amount of acid or base needed to adjust the pH may vary from one test solution concentration to the next, and the effect the test chemical has on pH may indirectly affect growth and development of the algae. Therefore, the pH of each test solution should be determined and compared to the acceptable range for growth and development of the test algae.

The data obtained in bioassays are usually expressed as standard response curves in which growth response of the test species is plotted against the concentration of the test chemical. The manner of expressing algal growth response varies considerably. For this guideline algal growth responses are expressed as direct measurements of number of algae per ml of solution. The statistical analysis (goodness-of-fit determination) facilitates accurate calculations of EC-10 and EC-50 as well as providing confidence limits for the concentration (dose)-response curve.

B. Test Conditions

1. Test Species

Both Salenastrum capricornutum and Skeletonema costatum have a number of useful characteristics as listed below,

which are necessary for an algal species to be used in bioassays (Toerien et al. 1971):

(a) broad nutrient response (grows both in oligotrophic and euthropic waters).
(b) distinct shape
(c) uniform size
(d) divide distinctly
(e) do not attach to glass or surface
(f) stay in suspension with slight agitation
(g) cells do not clump (aggregate)
(h) grow at a maximum rate in a short time in a medium simple to constitute
(i) do not excrete autotoxins
(j) cells are easy to count by both direct or indirect methods.

Selenastrum capricornutum is an excellent laboratory freshwater organism, easy to culture and count, and is both sensitive and consistent in its response to a wide range of nutrient levels (Payne and Hall 1979).

When included in multispecies toxicity screening tests, Selenastrum has been found to be a comparably sensitive species. Maki and Macek (1978) found this to be true in an environmental safety assessment for a nonphosphate detergent builder. Selenastrum was as sensitive to trinitrotoluene as the copepod, Trigriopus californicus, and was twice as sensitive as oyster larvae (Smock et al. 1976). Selenastrum was as sensitive as Daphnia and the fathead minnow to eight preparations of synfuels (Greene, personal communication). In a study of the toxicity of 56 dyes to Selenastrum and fish (fathead minnows), basic dyes do not markedly inhibit

algal growth, and "of special significance, however, is the rather startling correlation between results of algal assays and the results of fish bioassays" (Little and Chillingworth 1974). Greene (personal communiation) analyzed the results of this study and found the algae appear more sensitive than fish to 35 of the dyes tested while the fish were only more sensitive to seven of the dyes tested. In a recent test conducted on 35 chemicals on the EPA priority pollutant list by EG & G Bionomics (Parrish, personal communication), there were no significant differences in the EC-0's between Selenastrum and Skeletonema, Daphnia and bluegill fish, Lepomis macrochirus. Selenastrum was significantly more sensitive than sheepshead minnow. In another 2 tests EG & G performed for Monsanto Industrial Chemical Co. (1979a,b) evaluating two phthalate esters (Santicizer 60 and 711), Selenastrum was as sensitive as Microcystis aerugenosa, Navicula pelliculosa, Skeletonema costatum and Dunaliella tertiolecta. Palmer (1969) has extensively reviewed the algal literature and has ranked the 60 most pollution tolerant genera as reported by 165 authors. In comparing two green algae often used in algal toxicity testing, Chlorella and Scenedesmus to Selenastrum, great variation is found. Of the 60 genera, Scenedesmus was the fourth most tolerant, Chlorella was the fifth most tolerant, but Selenastrum was the fifty-seventh most tolerant. This analysis is borne out by recent results obtained by Green (personal communication) in testing effluent toxicity to algae. He found that Chlorella and Scenedesmus are generally more resistant to industrial effluents and both were naturally present in 100% effluents (eight submitted by

the USEPA Industrial Environmental Laboratory, Research Triangle Park, Raleigh, North Carolina). Selenastrum only grew when the effluents were diluted to 1-10% of the original concentration (which supported Chlorella and Scenedesmus growth). This was also the case in another effluent which contained 1.7 mg/l cyanide. Both Chlorella and Scenedesmus grew in it, but Selenastrum grew only when the effluent was diluted to 1% or less. Chlorella has also recently been shown to be much less sensitive to toxics than Daphnia or fish (Kenaga and Molenaar, 1979).

While it is recognized that numerous marine algae are sensitive to toxicants (North et al. 1972); heavy metals (Davies 1978), simple organics (benzene, cresol, hexane, phenol and toluene), various inorganics (Cl, CN, Hg) and complex wastes (industrial sewage, sulfite waste liquor, detergent), and petroleum compounds (Corner 1978), Skeletonema costatum was selected for use in the toxicity test guideline. This species has been frequently reported on in the bioassay literature (US Army 1978), and is a recommended bioassay organism (APH 1975, USEPA 1977a, b, 1978, Gentile and Johnson 1974).

The testing procedure for Skeletonema has recently proven useful for the evaluation of the relative potential hazards of a compound or a complex waste by providing data for the calculation of the EC-50 or SC-20 (Walsh and Alexander 1980, Walsh et al. 1980). Skeletonema was as sensitive to the 35 priority pollutants and two phthalate esters as Selenastrum in multi-species toxicity screening tests, as in the previously described studies.

Skeletonema was found to be more sensitive (at 10ppb) to growth inhibition effects induced by PCB's than two freshwater algae (Euglena gracilis and Chlamydomonas reinhardtii) and two other marine algae (Thalassiosira pseudonana, and Dunaliella tertiolecta) (Mosser et al. 1972).

Skeletonema costatum was also more sensitive (growth inhibited) at lower concentrations of wastewater chlorination products (3-chlorobenzoic acid, 5-chlorouracil, 4-chlororesorcinol, 3-chlorophenol and Captan) than Dunaliella tertiolecta and Porphyridium sp. (Sikka and Butler 1977).

Skeletonema and Selenastrum are specified for testing toxicity of pesticides (Subpart J, Pesticide Registration Guidelines). Additional justification for selection of these test species is provided in these guidelines (see FR 45 (214): 72948-72978).

Other species may be substituted for either of these two species when appropriate. Some freshwater or marine species which are of concern or have a significant ecological role may constitute a more crucial risk population. If so, those species of particular ecological or economic value should be selected. The rationale for selection of alternative species should be discussed with the Agency and/or supported in the report of findings.

2. Facilities

a. General

The test requires a growth chamber or temperature controlled enclosure capable of maintaining a uniform temperature of 24° $\pm$ 1°C if Selenastrum is tested or

20° ± 1°C if Skeletonema is tested. Other facilities typically needed include standard laboratory glassware, culture flasks, work areas to clean and prepare equipment and to measure chemical concentrations and algal growth and proper disposal facilities. Without these facilities, the testing cannot be adequately conducted.

b. Test Containers

Sterile Erlenmeyer flasks are recommended as test and culture containers. Any flask volume may be used between 125-500 ml. However, it is imperative that flasks of the same volume be used throughout the test. Hannon and Patouillet (1979) found a marked difference (2.6x) in mercury toxicity for marine algae, Phaeodactylum tricornutum, depending on the surface : volume ratio of the culture vessel. Flasks should be stoppered with sterile plugs (such as foam rubber or cotton stoppers) which will prevent possible bacterial contamination yet allow air flow.

c. Cleaning and Sterilization

Standard good laboratory practices are recommended to remove dust, dirt, other debris, and organic and inorganic residues from the test containers and other glassware and supplies should be washed and sterilized to prevent contamination.

Algal cells are discarded at the end of a test. Algae are capable of considerable adaptation to the toxic effects of antimetabolites and antibiotics, such as streptomycin, penicillin, chloramphenicol, sulfanilimide and sodium selenate (Kumar 1964).

It is important to avoid contamination of algal cultures by bacteria. Bacteria may metabolize high molecular weight

organic compounds to produce carbon dioxide and/or cofactors that stimulate growth of Selenastrum (Tison and Lingg 1977, Sachdev and Clesceri 1978). Consequently axenic cultures of algae should be maintained by proper sterile culture techniques as well as growing and testing algae in sterile containers and nutrient medium.

d. Conditioning

Test containers are to be rinsed with appropriate test solutions prior to the beginning of the toxicity tests. This method should allow for sorption of the test substance to the test container, thereby saturating the container surface so that no further interactions of test substance will take place when new test solution is added and the test begins. Hannan and Patouillet (1979) found that up to 50% of mercury could be lost to adsorption to vessel walls in a two-day toxicity test. Therefore, with proper conditioning all the test substance in the test solution should be available to test algae and any results will reflect an accurate concentration response.

e. Nutrient Medium

The nutrient medium recommended in the test guideline, are those currently recommended by the USEPA for use in bioassays (USEPA 1977, 1978a,b,c, Walsh and Alexander 1980, Walsh et al. 1980).

Use of the nutrient media under the test conditions will ensure maximum growth rates (i.e., logarithmic) in test algae and controls. Selenastrum and Skeletonema will divide 2-3 times per day (Nielsen 1978, Lewin and Guillard 1963, USEPA 1971b). This should enhance exposure of test algae to the test substance because algal cells in this growth phase

absorb and metabolize substances at a rapid rate (Fogg 1965). Shiroyama et al. (1973) found maximum phosphorus and nitrogen uptake occurred in the first five days of growth.

Many media used for culturing algae contain a chelating agent, usually EDTA, to keep micronutrients in solution. However, a medium containing a chelating agent is less than ideal for testing toxicants because chelators can increase or decrease toxicity and can add uncertainty to the test results (Payne 1975, Fogg 1965, Prakash and Rashid 1968, Bender 1970, Giesy 1974, Lin and Schelske 1979, Barber and Ryther 1969, Johnston 1964, Droop 1960, 1962; Eyster 1968, Erickson et al. 1970).

3. Environmental Conditions

Selenastrum and Skeletonema will grow over a wide temperature range, from less than 5°C to 35°C (Claesson and Forsberg 1978), and between 13°C and 30°C (Fogg 1965), respectively. The temperature selected for toxicity testing using Selenastrum was 24°C because luxury uptake of ammonia nitrogen, maximum specific growth rate, and sensitivity to phenol occur at that temperature (Reynolds et al. 1974, 1975a, 1975b 1976). The test temperature 20°C selected for Skeletonema is recommended in other toxicity testing manuals (USEPA 1978a,c) and in recent publications (Walsh and Alexander 1980, (Walsh and Alexander 1980, Walsh et al. 1980).

Algae require light for photosynthesis and growth. Fitzgerald (1975) and Miller et al. (1978) have shown that light intensity will affect the rate of growth of Selenastrum. As practically all the provisional algal assay procedure (Joint Industry/Government Task Force 1969)

development work was done on Selenastrum at 400 ft-c, it was not seen as necessary to make a change (USEPA 1978b). Continuous lighting of algal cultures is required for Selenastrum in the test guideline. While this does not reflect environmental conditions, it does maximize testing for toxicity. Practically all toxicity tests using Skeletonema have recommended split day/night lighting (USEPA 1978a, 1978c, Walsh and Alexender 1980, Walsh et al. 1980). For the sake of consistency, it was not seen as necessary to make a change in the procedure.

The test guideline requires a test solution pH of 7.5 for Selenastrum because it maximizes growth. Selenastrum grows between pH 4 and 10 (Brezonik et al. 1975) and maximally between pH 7 and 9.6 (Claesson and Forsberg 1978). Maximum adenosine triphosphate (ATP) (i.e., energy production) occurs in Selenastrum cultured between pH 7.5 and 8 (Brezonik et al. 1975). The pH selected for testing with Skeletonema, 8.1, was selected because it is recommended by other toxicity testing manuals (USEPA 1978a) and in recent publications (Walsh and Alexander 1980, Walsh et al. 1980) and approximates the natural oceanic pH. The pH should be adjusted as exactly as possible to the test pH because fluctuations in pH affects toxicity.

The purposes of oscillating the cultures are to enhance exposure of algal cells to test substances and to enhance dissolution and solubilization of test substances in the test solution. Turbulence created by shaking algal cultures is important to enhance the transfer of dissolved substances between the media and the cells. Munk and Riley (1952) showed that this transfer is faster if nutrients are

continually renewed adjacent to the cell by movement of the medium.

Oscillating test containers is also analogous to wind and wave induced mixing of natural waters. This agitation and mixing serves to maximize algal exposure to the test substance.

Temperature, light intensity, pH and oscillation rate are all recorded as specified in the test guideline to ensure that the environmental conditions of the test are met.

Temperature should be recorded at least hourly to ensure that it does not exceed the specified limits. Inexpensive growth chambers are available which are equipped with adequate recording instruments or chambers may be equipped with ones at minimal cost. Severe fluctuations in temperature may affect algal growth and/or subsequent chemical uptake or metabolism.

Light intensity readings at t e surface of the solutions may be made manually and ensure that all containers are receiving equal light. Light variations will affect algal growth so daily recordings are necessary to maintain uniform and constant radiation. The pH is measured at the beginning and end of the test as an indication of effects of test chemical additions and subsequent algal metabolism on the hydrogen-ion concentration. This will indicate if the test solution is outside of the algal pH optima for growth as well as show what pH variations may exist between chemical concentrations.

C. Reporting

The sponser should submit to the Agency all data developed during the test that are suggestive or predictive of phytotoxicity. If testing specifications are followed, the sponsor should report that specified procedures were followed and present the results. If alternative procedures were used instead of those recommended in the test guideline, then the protocol used should be fully described and justified.

Test temperature, chemical concentrations, test data, concentration-response curves, and statistical analyses should all be reported. The justification for this body of information is contained in this support document. If algal species other than the two recommended were used, the rationale for the selection of the other species should be provided.

III. Economic Aspects

The Agency awarded a contract to Enviro Control, Inc. to provide an estimate of the cost for performing an acute toxicity test using freshwater algae according to the Guideline. Enviro Control supplied two estimates; a protocol estimate and a laboratory survey estimate.

The protocol estimate was $1760. This estimate was prepared by identifying the major tasks needed to do a test and estimating the hours to accomplish each task. Appropriate hourly rates were then applied to yield a total direct labor charge. An estimated average overhead rate of 115%, other direct costs of $400, a general and administrative rate of 10%, and a fee of 20% were then added to the direct labor charge to yield the final estimate.

Environ Control estimated that differences in salaries, equipment, overhead costs and other factors between laboratories could result in as much as 50% variation from this estimate. Consequently, they estimated that test costs could range from $878 to $2636.

The laboratory survey estimate was $1465, the mean of the estimates received from eight laboratories. The estimates ranged from $430 to #3600 and were based on the costs to perform the test according to the Guideline.

Although a cost analysis was not performed for a test using marine algae, the procedures used are similar to the freshwater algal test and the costs should be similar.

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FISH ACUTE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
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FISH ACUTE TOXICITY TEST

(a) Purpose. This guideline may be used to develop data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (P.L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 et. seq.). This guideline prescribes tests to be used to develop data on the acute toxicity of chemicals to fish. The United States Environmental Protection Agency (EPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) Definitions. The definitions in Section 3 of the Toxic Substances Control Act (TSCA), and the definitions in "Good Laboratory Practice Standards for Physical, Chemical, Persistence, and Ecological Effects Testing" (Proposed Part 772, Subpart B, Section 772.110-2) apply to this test guideline. The following definitions also apply to this guideline:

(1) "Acclimation" means the physiological compensation by test organisms to new environmental conditions (e.g., temperature, hardness, pH).

(2) "Acute toxicity test" means a method used to determine the concentration of a substance that produces a toxic effect on a specified percentage of test organisms in a short period of time (e.g., 96 hours). In this guideline, death is used as the measure of toxicity.

(3) "Carrier" means a solvent used to dissolve a test substance prior to delivery to the test chamber.

(4) "Conditioning" means the exposure of construction materials, test chambers, and testing apparatus to dilution water or to test solutions prior to the start of a test in order to minimize the sorption of the test substance onto the test facilities or the leaching of substances from the test facilities into the dilution water or test solution.

(5) "Death" means the lack of opercular movement by a test fish.

(6) "Flow-through" means a continuous or an intermittent passage of test solution or dilution water through a test chamber, or a holding or acclimation tank with no recycling.

(7) "Incipient LC50" means that test substance concentration, calculated from experimentally-derived mortality data, that is lethal to 50 percent of a test population when exposure to the test substance is continued

until the mean increase in mortality does not exceed 10% in any concentration over a 24 hour period.

(8) "LC50" means that test substance concentration, calculated from experimentally-derived mortality data, that is lethal to 50 percent of a test population during continuous exposure over a specified period of time.

(9) "Loading" means the ratio of fish biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber or passing through it in a 24 hour period.

(10) "Static" means the test solution is not renewed during the period of the test.

(11) "Test solution" means the test substance and the dilution water in which the test substance is dissolved or suspended.

(c) Test procedures--(1) Summary of the test. (i) Test chambers are filled with appropriate volumes of dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired.

(ii) The test substance is introduced into each test chamber. In a flow-through test, the amount of test substance which is added to the dilution water is adjusted to establish and maintain the desired concentration of test substance in each test chamber.

(iii) Test fish which have been acclimated in accordance with the test design are introduced into the test and control chambers by stratified random assignment.

(iv) Fish in the test and control chambers are observed periodically during the test; dead fish are removed at least twice each day and the findings are recorded.

(v) The dissolved oxygen concentration, pH, temperature and the concentration of test substance are measured at intervals in selected test chambers.

(vi) Concentration-response curves and LC50 values for the test substance are developed from the mortality data collected during the test.

(2) [Reserved]

(3) Range finding test. If the toxicity of the test substance is not already known, a range finding test should be performed to determine the range of concentrations to be used in the definitive test. The highest concentration of test substance for use in the range finding test should not exceed its solubility in water or the permissible amount of the carrier used.

(4) Definitive test. (i) A minimum of 20 fish should be exposed to each of five or more test substance concentrations. The range of concentrations to which the

fish are exposed should be such that in 96 hours there are at least two partial mortality exposures bracketing 50% survival.

(ii) For exposure to each concentration of a test substance, an equal number of test fish should be placed in two or more replicate test chambers. The distribution of individual fish among the test chambers should be randomized.

(iii) Every test should include a control consisting of the same dilution water, conditions, procedures, and fish from the same group used in the test, except that none of the test substance is added.

(iv) Mortality data collected during the test are used to calculate a 96-hour LC50. The 24-, 48-, and 72-hour values should be calculated whenever there is sufficient mortality data to determine such values. If the 96-hour LC50 is less than 50% of the estimated 48-hour LC50 in a flow-through test, the test should be continued until the mean increase in mortality at any test concentration does not exceed 10% over a 24-hour period or until 14 days.

(v) Test fish should not be fed while they are being exposed to the test substance under static conditions or during the first 96 hours of flow-through testing. If the

test continues past 96 hours, the fish should be fed a suitable food at a maintainance level every other day beginning on test day 5. Any excess food and the fecal material should be removed when observed.

(5) Test results. (i) Death is the primary criterion used in this test guideline to evaluate the toxicity of the test substance.

(ii) In addition to death, any abnormal behavior such as, but not limited to, erratic swimming, loss of reflex, increased excitability, lethargy, or any changes in appearance or physiology such as discoloration, excessive mucous production, hyperventilation, opaque eyes, curved spine, or hemorrhaging should be recorded.

(iii) Observations on compound solubility should be recorded. The investigator should report the appearance of surface slicks, precipitates, or material adhering to the sides of the test chamber.

(iv) Each test and control chamber should be checked for dead fish and observations recorded at 24, 48, 72, and 96 hours after the begining of the test or within one hour of the designated times. If the test is continued past 96 hours, additional observations should be made every 24 hours until termination.

(v) The mortality data is used to calculate LC50's and their 95% confidence limits, and to plot concentration-response curves for each time interval whenever sufficient data exists. The methods recommended for use in calculating LC50's include probit, logit, binomial, and moving average angle.

(vi) A test is unacceptable if more than 10 percent of the control fish die or exhibit abnormal behavior during a 96-hour test. If a flow-through test is continued past 96 hours, the maximum allowable additional mortality is 10 percent.

(6) Analytical measurements--(i) Water quality analysis. (A) The hardness, acidity, alkalinity, pH, conductivity, TOC or COD, and particulate matter of the dilution water should be measured at the beginning of each static test and at the beginning and end of each flow-through test. The month to month variation of the above values should be less than 10% and the pH should vary less than 0.4 units.

(B) During static tests, the dissolved oxygen concentration, temperature and pH should be measured in each test chamber at the beginning of the test and as often as needed thereafter to document changes from the initial

levels. The test solution volume should not be reduced by more than 10% as a result of these measurements.

(C) During flow-through tests, dissolved oxygen, temperature and pH measurements should be made in each chamber at the beginning of the test and every 48 hours thereafter until the end of the test.

(ii) Collection of samples for measurement of test substance. Test solution samples to be analyzed for the test substance should be taken midway between the top, bottom, and sides of the test chamber. These samples should not include any surface scum or material dislodged from the bottom or sides. Samples should be analyzed immediately or handled and stored in a manner which minimizes loss of test substance through microbial degradation, photodegradation, chemical reaction, volatilization, or sorption.

(iii) Measurement of test substance. (A) For static tests, the concentration of dissolved test substance (that which passes through a 0.45 micron filter) should be measured at a minimum in each test chamber at the beginning (0-hour, before fish are added) and at the end of the test. During flow-through tests, the concentration of dissolved test substance should be measured as follows:

(1) in each chamber at 0-hour;

(2) in each chamber at 96-hours and every 4 days thereafter as long as the test is continued and;

(3) in at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system.

(B) Filters and their holders used for determining the dissolved test substance concentrations should be prewashed with several volumes of distilled water and undergo a final rinse with test solution. Glass or stainless steel filter holders are best for organic test substances, while plastic holders are best for metals. The sample should be filtered within 30 minutes after it is taken from the test chamber.

(C) The analytical methods used to measure the amount of test substance in a sample should be validated before beginning the test. The accuracy of a method should be verified by a method such as using known additions. This involves adding a known amount of the test substance to three water samples taken from a chamber containing dilution water and the same number and species of fish as are used in the test. The nominal concentration of the test substance in those samples should span the concentration range to be used in the test. Validation of the analytical method

should be performed on at least two separate days prior to starting the test.

(D) An analytical method is not acceptable if likely degradation products of the test substance give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test.

(E) In addition to analyzing samples of test solution, at least one reagent blank, containing all reagents used, should also be analyzed.

(F) If the measured concentrations of dissolved test substance are considerably lower (e.g. <50 percent) than the nominal concentrations, the total test substance concentration should be measured in the highest test concentration.

(G) Among replicate test chambers, the measured concentrations should not vary more than 20%. The measured concentration of the test substance in any chamber during the test should not vary more than 30 percent from the measured concentration prior to initiation of the test.

(H) The mean measured concentration of dissolved test substance should be used to calculate all LC50's and to plot all concentration-response curves.

(d) Test conditions--(1) Test species. (i) Selection. The test species for this test are the rainbow trout (Salmo gairdneri), bluegill (Lepomis macrochirus) and fathead minnow (Pimephales promelas). The particular species of fish to be used will be prescribed in the test rule.

(ii) Age and Condition of Fish. (A) Juvenile fish should be used. Fish used in a particular test should be the same age and be of normal size and appearance for their age. The longest fish should not be more than twice the length of the shortest.

(B) All newly acquired fish should be quarantined and observed for at least 14 days prior to use in a test.

(C) Fish should not be used for a test if they appear stressed or if more than five percent die during the 48 hours immediately prior to the test.

(iii) Acclimation of test fish. (A) If the holding water is not from the same source as the test dilution water, acclimation to the dilution water should be done gradually over a 48-hour period. The fish should then be held an additional 14 days in the dilution water prior to testing. Any changes in water temperature should not exceed $3^{o}C$ per day. Fish should be held for a mininum of 7 days at the test temperature prior to testing.

(B) During the final 48-hours of acclimation, fish should be maintained in facilities with background colors and light intensities similar to those of the testing area and should not be fed.

(2) Facilities--(i) General. Facilities needed to perform this test include:

(A) flow-through tanks for holding and acclimating fish,

(B) A mechanism for controlling and maintaining the water temperature during the holding, acclimation and test periods,

(C) Apparatus for straining particulate mater, removing gas bubbles, or insufficient dissolved oxygen, respectively,

(D) Apparatus for providing a 16 hour light and 8 hour dark photoperiod with a 15- to 30-minute transition period,

(E) Chambers for exposing test fish to the test substance;

(F) A test substance delivery system for flow-through tests.

(ii) Construction materials. Construction materials and commercially purchased equipment that may contact the stock solution, test solution, or dilution water should not contain substances that can be leached or dissolved into

aqueous solutions in quantities that can alter the test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test chemicals. Glass, #316 stainless steel, and perfluorocarbon plastic should be used whenever possible. Concrete, fiberglass, or plastic (eg.PVC) may be used for holding tanks, acclimation tanks, and water supply systems, but they should be used to remove rust particles. Rubber, copper, brass, galvanized metal, epoxy glues, and lead should not come in contact with the dilution water, stock solution, or test solution.

(iii) Test substance delivery system. In flow-through tests, diluters, metering pump systems or other suitable devices should be used to deliver the test substance to the test chambers. The sysstem used should be calibrated before each test. Calbration includes determining the flow rate through each chamber and the concentration of the test substance delivered to each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow rate through a test chamber. During a test, the flow rates should not vary more than 10 percent from one test chamber to another or from one time to any other.

(iv) Test chambers. Test chambers made of stainless steel should be weled, not soldered. Test chambers made of glass should be fused or bonded using clear silicone adhesive. As little adhesive as possible should be left exposed in the interior of the chamber.

(v) Cleaning of test system. Test substance delivery systems and test chambers should be cleaned before each test. They should be washed with detergent and then rinsed in sequence with clean water, pesticide-free acetone, clean water, and five percent nitric acid, followed by two or more changes of dilution water.

(vi) Dilution water. (A) Clean surface or ground water reconstituted water, or dechlorinated tap water is acceptable as dilution water if the test fish will survive in it for the duration of the holding, acclimating, and testing periods without showing signs of stress, such as discoloration, hemorrhaging, disorientation or other unusual behavior. The quality of the dilution water should be constant and should meet the following specifications measured at least twice a year:

Substance	Maximum Concentration
Particulate matter	20 mg/liter
Total organic carbon or	2 mg/liter
chemical oxygen demand	5 mg/liter
Un-ionized ammonia	lug/liter
Residual chlorine	1 ug/liter
Total organochloring pesticides	50 ng/liter
Total organocholorine pesticides plus polychlorinated bephenyls (PCBs) or organid chlorine	50 ng/liter 25 hg/liter

(B) The concentration of dissolved oxygen in the dilution water should be between 90 and 100 percent saturation; 9.8-10.9 mg/l for tests with trout, and 8.0-8.9 mg/l for tests with bluegill or fathead minnow at sea level. If necessary, the dilution water can be aerated before the addition of the test substance. All reconstituted water should be aerated before use. Buffered soft water should be aerated before but not after the addition of buffers.

(C) If disease organisms are present in the dilution water sufficient numbers to cause infection, they should be killed or removed by suitable equipment.

(D) Glass distilled or carbon filtered deionized water with a conductivity less than 1 micromho/cm is acceptable for use in making reconstituted water. If the reconstituted water is prepared from a ground or surface water source, conductivity, and total organic carbon (TOC) or chemical oxygen demand (COD) should be measured on each batch.

(vii) Carriers. (A) Distilled water should be used in making stock solutions of the test substance. If the stock volume however is more than 10% of the test solution volume, dilution water should be used. If a carrier is absolutely necessary to dissolve the test substance, the volume used should not exceed the minimun volume necessary to dissolve or suspend the test substance in the test solution. If the test substance 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.

(B) Triethylene glycol and dimethyl formamide are the perferred cariers, but acetone can also be used. The concentration of triethylene glycol in the test solution should not exceed 80 mg/l. The concentration of dimethyl formamide or acetone in the test solution should not exceed 5/0 mg/l.

(3) Test parameters--(i) Loading. The number of fish placed in a test chamber should not be so great as to affect the results of the test. The loading should not be so great that the test substance concen- trations are decreased by more than 20 percent due to uptake by the fish. In static tests, loading should not exceed 0.5 grams of fish per liter of solution in the test chamber at any one time. In flow-through tests loading should not exceed 0.5 grams of fish per liter of test solution passing thrbugh the chamber in 24 hours. These loading rates should be sufficient to maintain the dissolved oxygen concentration above the recommended levels and the ammonia concentration below 20 ug/l.

(ii) Dissolved oxygen concentration. (A) During static tests with rainbow trout the dissolved oxygen in each test chamber should be greater than 5.5 mg/l. In tests with bluegill and fathead minnows, the DO should be maintained above 4.5 mg/l.

(B) During flow-through tests the dissolved oxygen concen- tration should be maintained above 8.2 mg/l in tests with trout and above 6.6 mg/l in tests with bluegills or fathead minnows.

(iii) Temperature. The test temperature should be 22 $\pm 1^{o}C$ for bluegill and fathead minnows, and $12 \pm 1^{o}C$ for

rainbow trout. The temperature should be measured at least hourly in one test chamber.

(iv) Light. A 16-hour light and 8-hour dark photoperiod with a 15- to 30-minute transition period should be maintained.

(e) Reporting. The sponsor should submit to the EPA all data developed by the test that are suggestive or predictive of toxicity. In addition to the reporting requirements prescribed in the Good Laboratory Practice Standards for Physical, Chemical, Persistence, and Ecological Effects Testing, the reported test data should include the following:

(A) The source of the dilution water, a description of any pretreatment, and the measured hardness, acidity, alkalinity, pH, conductivity, TOC or COD and particulate matter.

(B) A description of the test chambers, the depth and volume of solution in the chamber, the specific way the test was begun (e.g., conditioning, test substance additions), and for flow-through tests, a description of the test substance delivery system.

(C) Detailed information about the test fish, including the scientific name and method of verification, average

weight (grams, wet weight), standard length, age, source, history, observed diseases, treatments and mortalities, acclimation procedures, and food used.

(D) The number of replicates used, the number of organisms per replicate, the loading rate, and the flow rate for flow-through tests.

(E) The measured DO, pH and temperature and the lighting regime.

(F) The solvent used,the test substance concentration in the stock solution, the highest solvent concentration in the test solution and a decription of the solubility determinations in water and solvents if used.

(G) The concentration of the test substance in each test chamber just before the start of the test and at all subsequent sampling periods.

(H) The number of dead and live tests organisms, the percentage of organisms that died, and the number that showed any abnormal effects in the control and in each test chamber at each observation period.

(I) The 96-hour LC50, and when sufficient data have been generated, the 24-, 48-, 72-, and incipient LC50 values, their 95 percent confidence limits, and the methods used to calculate the LC50 values and their confidence limits.

(J) When observed, the observed no effect concentration (the highest concentration tested at which there were no mortalities or abnormal behavioral or physiological effects).

(K) The concentration-response curve at each observation period for which a LC50 was calculated.

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

ES-6
August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

FISH ACUTE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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Technical Support Document for Fish Acute Toxicity Test

I. Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-9 which uses rainbow trout, bluegill or fathead minnow to evaluate the acute toxicity of chemical substances to fish. The Document provides an account of the scientific evidence and an explanation of the logic used in the selecion of the test methodology, procedures and conditions prescribed in the Test Guideline. Technical issues and practical considerations are discussed. In addition, estimates of the cost of conductiing the test are provided.

II. Scientific Aspects

A. Test Procedures

1. Range Finding Test

A range finding test is recommended for determining the appropriate concentractions of test substance to use in performing a definitive acute toxicity test. In the range finding test, groups of five or more test fish are exposed to a broad range of concentrations of the substance. Enough concentrations should be tested such that concentration lethal to approximately 50% of the organisms can be ascertained. The number of concentrations will normally range from 3-6 depending upon the shape of the toxicity curve for that chemical and prior knowledge of its approximate toxicity. Only concentrations less than the solubility limit in water are tested. The exposure period used in the range finding test can be as short as 24 hours or as long as 96 hours. If an exposure period less than 96 hours is used, the test substance concentration range

selected for the definitive test may have to be adjusted to make allowances for a greater potential toxicity after 24-hours of exposure. If no mortalities at a test concentration equal to the solubility limit are produced, no additional higher concentrations need to be tested.

2. Definitive Test

a. General

The results of a definitive test are used to calculate the 96-hour LC50 and the incipient LC50 when appropriate, and the concentration-response relationship of the test substance and the test fish. If the concentrations of test substance which produce no effect, a partial kill, and 100 percent mortality have been determined during the range finding test, then five or six test substance concentrations should be sufficient to estimate the appropriate LC50 values in a definitive test. In some cases however, to obtain two partial kills bracketing the 50% mortality level, it may be necessary to test 8-10 concentrations.

The slope of the concentration-response curve provides an indication of the range of sensitivity of the test fish to the test substance and may allow estimations of lower concentrations that will affect the test organisms. For example if the slope of the concentration-response curve is very steep, than a slight increase in concentration of the test substance will affect a much greater portion of the test fish than would a similar increase if the slope of the curve was very shallow. The slope of the concentration-response curve reveals the extent of sensitivity of the test fish over a range of concentrations.

The exposure of a minimum of 20 fish, divided into two

or more replicate groups , to each test substance concentration is required in the guideline. That minimum is based on an optimum number of test fish needed for statistical confidence, equipment requirements, and practical considerations of handling the test organisms.

At least two replicates should be included in order to demonstrate the level of pecision in the data and indicate the significance of variations. Test chambers holding replicate groups should have no water connections between them. The distribution of test fish to the test chambers should be randomized to prevent bias from being introduced into the test results.

Fish should not be fed during the test for two reasons. First, fecal matter which may accumulate can result in a decrease in the dissolved oxygen concentration in the test chamber. Second, some test substances can physically bind to the uneaten food or fecal matter, thus making a portion of the test substance unavailable for uptake by the fish. An occurrence of either of these conditions could produce unreliable test data.

b. Time-dependent vs. Time-independent

In time-dependent tests, fish are continuously exposed to a series of concentrations for a specified period of time, usually 96-hours, at which time the LC50 is calculated. In time-independent tests (TI's), fish are continously exposed to a series of test concentrations until such a time when no additional mortalities are expected to result from continued exposure. The LC50 calculated at this time is termed the incipient LC50. This same value has also been termed the ultimate median tolerance limit, the lethal

threshold concentration or the asymptotic LC50 by many researchers. Sprague (1969) has stated that the incipient LC50 is the "most useful single criterion of toxicity." In a review of 375 toxicity tests by the author, a lethal threshold clearly had not been reached in 42 tests while in 122 tests th threshold was reached in four days or longer. Eat n (1970) proposed the use of TI's as the resulting 50% effect concentration is dependent upon the response of the test fish and not based on an arbitrary time period.

Use of TI's greatly increases one's ability to evaluate the chronicity of compounds as discussed by Tucker and Leitzke (1979). The chronicity of a compound, or the degree to which a compound effects additional mortalities over a prolonged period of time, is assessed by comparing LC50's over time, e.g. 96/48 hour LC50 or 10 day incipient LC50/96 hour LC50. Those compounds with relatively high ratios (i.e. >0.5) would not be expected to cause chronic effects. If only 96-hour toxicity tests were performed, the toxicity of those compounds whose mode of action requires at least 3-4 days to begin to express toxicity would be grossly underestimated.

It should be remembered that for compounds that do not express chronicity, i.e. there is little additional mortality during the last 48 hours of the test, the test will be a 96-hour toxicity test. Consequently, the more costly testing for the estimation of an incipient LC50 will be performed only for compounds for which additional testing will probably be performed during the hazard or risk assessment process.

c. Static vs. Flow-through

In the static system, the test substance and dilution water are not changed during the 96-hour exposure period. In promulgating test rules, the Agency will prescribe the use of a static or flow-through system, or both, for fish acute toxicity tests for developing, cost effectively, those data essential to assessing the risk to the environment of a given chemical.

Each of these methods of exposing fish to a test substance offers certain advantages and presents certain disadvantages not shared by the other. The static exposure system requires less equipment and set up time, and therefore is a less expensive test. On the other hand, in a flow-through system, loss of the test substance due to uptake by the fish, degradation, or to volatilization is minimized, and metabolic products toxic to the test organisms (e.g. ammonia) do not build up. The concentration of dissolved oxygen in the test chamber can also be maintained above the level that might stress the fish.

Because of these features, the Agency will specify the use of the flow-through method in testing the toxicity of chemicals which volatilize or degrade rapidly, which reduce the dissolved oxygen concentration within the test chamber, or which are taken up by the test organisms at a rate that significantly lowers the concentration of the test substance within the test chamber. In additon to developing data needed to determine the 96-hour LC50 and the concentration-response curve for such test substances, a flow-through exposure may be continued to get information on the potential chronicity of the compound. By design, static

tests can not be continued past 96 hours and still yield reliable results.

In a static system, since the test substance is not renewed, the concentration of the chemical to which test fish are exposed does not remain constant if the chemical adheres to the test chamber walls, vaporizes readily, degrades rapidly, or is readily taken up by the test fish.

The static method of exposure however, can be used to develop toxicity data for those substances which are not subject to a significant reduction in concentration during the exposure period. Static toxicity data in combination with data developed through the use of a flow-through test can also be used to detect and evaluate the toxicity of metabolities and degradation products. If for instance, the 96-hour LC50 from a static test is less than that from a 96 hour flow-through test, it can be assumed that more toxic metabolites or degradation products were formed during the static test.

The Agency forsees a need for both flow-through and static test methods and each method will be considered in the developement of a test rule. The chemical nature of the test substance, its use and the nature of its release into the environment (e.g.,continuous or intermittent) will be considered. The Agency does not, however, assume that data developed through the use of one of these test methods can substitute for data developed through the use of the other, since evidence exists in the literature to show that the toxicity of some test substances for test organisms may be 10 times greater in flow-through than in static exposures (Mauck et al. 1976).

d. Length of Exposure

The minimium exposure time of 96 hours in the fish acute toxicity test standard is specified in order to permit a comparison of data developed through the use of this test guideline with the large base of acute toxicity data in the published literature. The 96-hour exposure period is advocated by various groups concerned with establishing uniformity in testing methodology (APHA 1975, ASTM 1980, Committee on Methods... 1975). Most of the recently published data on the acute toxicity of chemicals to freshwater fish were developed using a96-hour exposure period (Brugns et al. 1977, McKim et al. 1976, Spehar et al. 1979, 1980). The use of the 96-hour exposure period was proposed initially in 1951 by an aquatic bioassay committee (Doudoroff et al. 1951) and was selected, in large part, as a matter of convenience since it is easily scheduled within the five-day work week. Only when there are indications of chronicity during a 96-hour test will the test period be extended. The previously cited studies indicate that this is not a frequent occurrence.

3. Test Results

While death is the primary endpoint in these tests, any behavioral or physiological changes in the fish such as erratic swimming, lost of reflexes,increased excitability, lethargy, discoloration, excessive mucous production, hyperventilation, opaque eyes, curved spine, hemorrhaging or any other observed effects should be recorded. Quantification of such observations at test substance concentrations not causing lethality are useful in identifying and assessing potential chronic lethal effects

at these lower test concentrations.

Mortality data at 24, 48, 72 and 96 hours and at termination if the test exceeds 96 hours, should be presented for each time interval. Whenever sufficient data exists at these time intervals,an LC50 and 95% confidence interval should be calculated and a graph of percentage mortality - concentration prepared. When more than one LC50's at various time intervals should be prepared.

The recommended methods of LC50 calculation include the probit, logit, binominal and moving average angle methods. The data obtained from each test will determine which method is the most appropriate for that data set.

4. Analytical Measurements

a. Water Quality Analysis

Measurement of certain water quality parameters of the dilution water such as hardness, particulate matter, alkalinity, acidity, conductivity, TOC, and pH is important. Quantification of these parameters at the beginning and end of the exposure period of flow-through tests is necessary in order to determine if the water quality varied during the test. If significant variation occurs, the resulting data should be interpreted in light of the estimated toxicity values.

In Static Systems the dissolved oxygen concentration and pH should be measured in each test chamber at the beginning of the test just prior to addition of the fish and then as often as needed to document any subsequent changes from the initial levels.

In Flow-through the dissolved oxygen concetration (DO) and pH of the test solution in each chamber should be

determined at the beginning of the test and every 48 hours thereafter until the end of the test. A decrease in DO indicates that the flow rate should be increased.

b. Collection of Test Solution Samples

The objective of the recommended sampling procedure is to obtain a representative sample of the test solution for use in measuring the concentration of the test substance. Although there is mixing in the test chamber, especially in flow-through tests, material can concentrate near the sides and bottom of the chamber due to physical or chemical properties of the substance, or to interactions with organic material associated with the test animals. For this reason, water samples should be taken near the center of the test chamber. The handling and storage of the samples requires care to prevent the loss of the test substance from the sample before analysis.

c. Test Substance measurement

In Static Systems the concentration of dissolved test substance should be measured in each test chamber at least at the beginning and end of each test. If the reduction in test substance concentration exceeds 50%, the test should be repeated at a lower loading rate, or a flow-through test should be performed.

In Flow-through Systems the test substance concentration should be determined in each test chamber at 0 and 96 hours and if the test continues past 96 hours, at least every four days up to and including the day of termination. To further assess and quantify any possible changes in test substance concentration, whenever a malfunction of the toxicant delivery is detected, all potentially affected test chambers

should be sampled at that time.

If the measured concentrations of dissolved test substance are 50 percent more or less than the nominal concentrations, steps should be taken to determine the cause for this deviation. A sample of the stock solution as well as samples of the influent to various test chambers should be analyzed to determine if the reduction in test substance occurs prior to delivery of the test solution to the aquaria. If results of these analyses indicate that the proper amounts of test substance are entering the test chambers, then the total test substance concentration should be measured in at least the chambers containing the highest test substance concentration. These data will give indications if the difference between nominal and measured test concentrations is due to volatilization or degradation of the test substance, or to insolubility of the test substance in the dilution water.

If the toxicant delivery system has been properly calibrated and the fish randomly introduced into each test chamber, the measured differences between replicates at each concentration should be less than 20%. If the differences exceed this, the test should be repeated.

The concentrations of test substance measured after initiation should be within 30% of the concentrations measured prior to introduction of the fish. If the difference exceeds this, the test should be repeated using a higher flow rate.

Use of reliable and validated analytical techniques and methods is essential to the usefulness of the test data in assessing the environmental hazard of the chemical.

Significant variation in the measured concentrations lessens the value of the toxicity data generated.

B. Test Conditions

1. Test Species

a. Selection

The species of fish selected for use in this test guideline are the rainbow trout, bluegill and fathead minnow. Adelman and Smith (1976) listed the following as the important criteria to use in the selection of a "standard fish" for bioassays: 1) relatively constant response to a broad range of toxicants when tested under similar conditions 2) available in large quantities with close quality control 3) easily handled for bioassay purposes 4) easily transported 5) continuous availability of the desired size and 6) capable of successful completion of a life cycle in 1 year or less. These species meet all criteria but the last; only the fathead minnow can complete a full life cycle in less than one year.

The main reason why these three species were selected is because there is a very large toxicity data base with each, and all three are readily available and require little expertise in maintaining healthy populations. All three are widely distributed in the United States, and are either ecologically or economically important (Scott and Crossman 1973, Kitchell et al. 1979).

Studies on relative sensitivity of the three species have been performed and indicate that rainbow trout are generally the most sensitive and fathead minnow the least sensitive to a variety of test substances. Kenage (1979) compiled LC50 data on 20 pesticides with all three

species. With 12 of these compounds, the trout was the most sensitive and the fathead minnow the least. In five cases the bluegill was the most sensitive and in one case the fathead minnow was the most sensitive. The LC50's were similar between the three species for the remaining two compounds. The LC50's for the fathead minnows were generally 6x those for the trout and 2x those for bluegill. As it was not stated if the tests were performed under comparable conditions these values only approximate relative sensitives of the species.

Nevins and Johnson (1978) tested three phosphate ester mixtures with all three species of fish under identical static conditions and two compounds under flow-through conditions. In all five cases, rainbow trout were the most sensitive, but only in 2 cases were the fathead minnows the least sensitive.

Folmar et al. (1979) performed static acute toxicity tests with technical grade glyphosate, the formulated pesticide Roundup® surfactant with the above 3 fish species. In these tests however, the fathead minnow was the most sensitive.

From these data it is clear that it can not be assumed that rainbow trout will be the most sensitive species. Although fathead minnows are generally the least sensitive of the three species, their small size, ease of culture, and short life cycle make them the easiest to work with. Their extensive use in early life stage testing and full-chronic testing (Macek and Sleight 1977, McKim 1977) adds to the importance of their role is aquatic toxicology programs. Although rainbow trout can also be used in early life stage

and full or potential chronic toxicity testing, the expense is only occasionally warranted.

b. Sources

All three species are readily available at the appropriate test sizes from commercial fish suppliers. There are some suppliers that now specialize in culturing fish under controlled conditions just for toxicity testing. Rainbow trout can be purchased and readily shipped to researchers as either eyed eggs or as fingerlings already the appropriate size for testing. Trout should oe purchased only from suppliers that have been state-certified to raise disease-free fish.

There are many suppliers of bluegill throughout the country that will readily air-freight fish. As these fish are not amenable to artificial fertilization or spawning in the laboratory, figerlings are normally shipped. Researchers need to carefully select their suppliers, as many are known to have little concern for providing disease and parasite-free fish.

Fathead minnows can be purchased as eggs or juveniles, or cultured in the laboratory in a brood unit (U.S. EPA 1971). In light of past problems with the health of fish received from some suppliers, it is recommended that researchers rear their own fatheads for toxicity testing.

Whenever fish are to be used for a test or a set of tests, all fish used for that test should be from the same source and held under similar conditions prior to testing to minimize variability. Alexander and Clarke (1978) performed toxicity tests with 2 strains of rainbow trout exposed to 13 mg/l dodecylsodium sulfate. The median survival time of the

Idaho strain exposed to phenol was significantly less than the median survival time of the Nisqually strain. Response times for the other compounds were similar between strains. In a second set of tests with just phenol, no difference in LC50 values was found between Idaho and Nisqually strains, but a significant difference was found between Idaho and Manx strains.

2. Maintenance of Test Species

a. Age and Condition

The age, and consequently the size of rainbow trout, bluegill, and fathead minnows was selected based on the ease of handling and testing fish of this size. All fish used in the same test should be as similar in size as possible to limit the effects due to size differences.

The health and condition of fish used in acute toxicity tests is an important consideration. Diseased or stressed fish may increase the sensitivity of the fish to the toxicant. Iwama and Greer (1980) performed 96-hour acute toxicity tests with Coho Salmon (Oncorhynchus kisutch) that had been exposed to, and contracted, a mild state of bacterial kidney disease (Conynebacterium salmoninus). When diseased fish were exposed to pentachlorophenate, the estimated LC50 was 39 ug/l, significantly lower than the LC50 of 65 ug/l for healthy fish.

Prior exposure to contaminants may also effect the response of test fish to a toxicant. Bills et at. (1977) performed acute toxicity tests with several compounds using rainbow trout that had previously been exposed to PCB's (Aroclor 1254). Previously exposed trout that had body burdens of 3.4 ug/g of Aroclor 1254 had significantly lower LC50's when exposed to two of the test compounds,

cyanide and chromium. Trout with body burdens of only 0.46 ug/l of PCB's were more sensitive to cyanide with a LC50 of 66 ug/l versus 90 ug/l for "clean fish."

Alexander and Clarke (1978) performed static acute toxicity tests with rainbow trout that had been exposed to 40 ug/l of total residual chlorine for 24 hours immediately prior to testing with phenol. Trout previously exposed to chlorine had a 48 hour LC50 of 7.7 mg/l for phenol, significantly lower than the LC50 of 10.1 mg/l for non chlorine- exposed trout.

Based on these data it is recommended that all fish used in aquatic toxicity testing contain no more than 0.5 ug/l PCB, and not be exposed to any contaminants during holding.

b. Care and Handling

Upon arrival at the laboratory, fish or eggs should immediately be cared for to prevent additional stress from crowding during transport. The test organisms should be gradually transferred to the holding water at the testing facility as soon as possible.

Alexander and Clarke (1978) performed acute toxicity tests with rainbow trout and five different potential reference toxicants to determine what effects starvation, changes in temperature, and crowding would have on the median survival time (MST) for each toxicant. Trout that were starved for 15-21 days before separate exposures to phenol and sodium pentachlorophenate had significantly lower MST's than fed fish. There were no differences in MST's among fish exposed to sodium azide, copper sulfate, or dodecylsodium sulfate. When trout that had been held at 10°C were subjected to temperature decreases of 1-5°C over

24 hours and then immediately transferred to test chambers at 15°C, only fish exposed to phenol had a significantly lowe MST than fish gradually acclimated to 15°C. Temperature stress did not alter the MST for the remaining four compounds.

To determine the potential effects of crowding, one group of trout was held at a high density of 3.3 g/l day, compared to the normal holding density of 0.6 g/l day. Only fish exposed to sodium azide and sodium pentachlorophenate had significantly lower MST's due to crowding.

In subsequent toxicity tests with just phenol, the authors determined that trout with holding mortalities as high as 7-9% and 18% had LC50's similar to those generated with trout with only a 1-2% holding mortality.

Although the above research does not present conclusive evidence on the role of feeding, crowding, and temperature changes during holding, it does demonstrate that at least for some test substances these variables should be controlled and optimized to prevent possible differences in test fish response to toxicant exposure.

c. Acclimation

Brauhn and Schoettger (1975) found that fish that had become accustomed to unrestricted swimming in rearing ponds underwent intense competition for food and swimming space when placed in confined holding tanks. Rainbow trout appeared to be less affected by restricted space than bluegill and fathead minnows. The authors also recommended that fish be maintained in holding tanks with color backgrounds and light intensitites similar to those in the testing area to prevent additional stress when transferred

to testing chambers.

Although the importance of temperature acclimation to the test temperature is still unclear, a maximum gradual change of 3°C/day is recommended at this time. Peterson and Anderson (1969) concluded that complete acclimation to temperature, based on changes in locomotor activity and oxygen consumption, requires approximately two weeks before metabolism is back to normal. They also determined that the rate of change was more important than the amount of change.

Changes in the hardness of water to which the fish are exposed should also be controlled. Lloyd (1965) determined that trout transferred from hard to soft water needed at least 5 days of acclimation to the soft water before their response to a toxic metal was the same as the response of fish continually held in soft water.

3. Facilities

a. General

Facilities needed to perform this test include: (1) flow-through tanks for holding and acclimating fish, (2) a mechanism for controlling and maintaining the water temperature during the holding, acclimation, and test periods, (3) apparatus for straining particulate matter, removing gas bubbles, or aerating the water when water supplies contain particulate matter, gas bubbles, or insufficient dissolved oxygen, respectively, (4) an apparatus for providing a 16-hour light and 8-hour dark photoperiod with a 15- to 30-minute transition period, (5) chambers for exposing test fish to the test substance, and (6) a test substance delivery system.

Flow-through tanks, into which a continuous or intermittant flow of water occurs, should be used for holding and acclimating test fish. The renewal of the water in flow-through tanks minimizes the accumulation of metabolic products such as ammonia. The build up of organic matter within the tanks might provide a nutrient source for bacteria present in the water. Bacteria using oxygen to metabolize and decompose the organic matter in the tank could then reduce the dissolved oxygen concentration of water. Decreased dissolved oxygen concentrations as well as the accumulation of ammonia could increase the likelihood of disease in the test fish (Brauhn and Schoettger 1975). The use of diseased fish in acute toxicity tests could result in the development of inaccurate and unreliable data.

The effects of sudden temperature changes on fish may range from death to temporary impairment of physiological functions, depending on the acclimation temperature, the magnitude of the temperature change, the temperature tolerance of the species, and the circumstances and duration of the exposure. To avoid any undue stress, accurate temperature control devices should be used to both maintain constant temperatures, and to gradually increase or decrease the temperature during acclimation procedures. Such mechanisms have been described by DeFoe (1977) and Lemke and Dawson (1970).

Particulate matter and gas bubbles, if present in the dilution water, may clog the toxicant delivery system used in flow-through tests. Gas bubbles also may cause excessive loss of volatile test substances. Either circumstance may alter the concentration of test substance to which the test

fish are exposed. To avoid this problem an apparatus capable of removing particulate matter or gas bubbles from the dilution water may be required. If the dilution water is heated prior to use,it may also be necessary to desaturate the water from >100% of oxygen saturation. Penrose and Squires (1976) describe a suitable apparatus for this.

An adequate supply of dissolved oxygen should be available to the fish. To facilitate this, the dilution water or holding water should be at 90- 100% of oxygen saturation prior to delivery to the holding tanks or test system.

The duration, and intensity of light are environmental variables which could possibly influence the results of acute toxicity tests. Any possible variations in test data due to differences in light conditions can be minimized by using uniform light conditions during testing. A device capable of regulating photoperiods and the transitions from light to darkness and darkness to light has been described by Drummond and Dawson (1979).

b. Construction Materials

Due to the toxicity of many heavy metals at low concentrations (U.S. EPA 1976) and the ability of metal pipe, galvanized sheeting, laboratory equipment, etc. to leach metals into water, no metal other than stainless steel (preferably #316) should be used. In the same manner, unaged plasticized plastic (Tygon® tubing) should not be used due to the high toxicity of a main component, di-2-ethyl hexyl phthalate (Mayer and Sanders 1973) and the abilitiy of DEHP to leach into aquaria systems from plastics (Carmignani and Bennett 1976). To avoid any possible stress due to

exposure to low levels of metals, phthalates, and other potential contaminants, #316 stainless steel, glass and perfluorocarbon plastics (e.g. Teflon®) should be used whenever possible and economically feasible. If other materials should be used, conditioning to a continuous flow of heated dilution water should be performed for a minimum of 48 hours.

c. Test Substance Delivery System

To maximize the accuracy and precision of test results developed through the use of this test guideline, the quantity of test substance introduced by the test substance delivery system should be as constant as possible from one addition of test substance to the next. Fluctuations in the quantity of test substance introduced into the test chamber may result in abnormally high or low response value (e.g. LC50's) of the test organisms and in a wider spread of response values in replicate tests. The greater the variation in the quantity of test substance introduced, the greater the potential for abnormalities and spread of the response values.

Variations in the quantity of dilution water entering the test chambers during a given time interval may also create undersirable differences in test conditions between test chambers. The concentrations of dissolved oxyen and test substance in a test chamber, for example, may decrease more rapidly in chambers having lower flow rates. Differences between test chambers in the concentration of dissolved oxygen, test substance, metabolic products and degradation products, individually or in combination may result in response values for the test organisms which are inaccurate.

Any one of several toxicant delivery devices can be used as long as it has been shown to be accurate and reliable. Various modifications of proportional diluters have been used by Auwarter (1977), DeFoe (1975), Mount and Brungs (1976), and Ozburn and Smith (1977). A manual for their construction and operation has been prepared by Lemke et al. (1978). A metering pump system has also been used by Chandler and Partridge (1975), as have saturator systems (Krugel et al. 1978, Veith and Comstock 1975).

The following criteria presented by Hodson (1979) should be considered when selecting or designing a toxicant delivery system; 1) the delivery of the toxicant should stop if delivery of the dilution water stops, 2) it should be consistent in delivery amounts throughout the test period, 3) independent of electrical failure, 4) independent of temperature and humidity fluctuations, 5) capable of delivering small quantities, 6) easy to construct with few moving parts and 7) easy to operate.

The solubility of the test compound should also be taken into account in selecting an appropriate delivery system. If the compound can be solubilized in water, a device capable of delivering amounts of test solution greater than 1 millilter (ml) will probably be needed. If a carrier should be used, a system capable of accurately delivering small amounts, less than 100 microliters (ul), will probably be required to minimize the carrier concentration in the test solution.

Each system should be calibrated prior to starting the test to verify that the correct proportion of test substance to dilution water is delivered to the appropriate test chambers.

d. Test Chambers

Test chambers should be constructed of stainless steel, perfluorocarbon plastic, or glass. Stainless steel should be welded, and the glass bonded with silicone adhesive. If adhesive is used, the amount exposed to the test solution should be minimized to limit the sorption of test materials. The size, shape and depth of the test chambers are not imporant, as long as the volume accomodates the loading requirements. The chamber however, should be sufficiently large and contain enough water such that the fish are not stressed due to crowding.

e. Cleaning of the Test System

Before use, test systems should be cleaned to remove dust, dirt, and any other debris or residues that may remain from previous use of the system. Any of these substances may affect the results of a test by sorption of test materials or by exerting an adverse effect on test organisms. New chambers should be cleaned to remove any dirt or chemical residues remaining from manufacture or accumulated during storage. Detergent is used to remove hydrophobic or lipid-like substances. Acetone is used for the same purpose and to remove any detergent residues. It is important to use pesticide-free acetone to prevent the contamination of the chambers with pesticides which influence the outcome of the test. Nitric acid is used to clean metal residues from the system. A final thorough rinse with water washes away the nitric acid residues. At the end of a test, test systems should be washed in preparation for the next test. It is easier to clean the equipment before chemical residues and organic matter become

embedded or absorbed into the equipment.

Conditioning the flow-through system with dilution water before it is used in a test allows an equilibrium to be established between any substance in the water and the materials of the test system. Since the test system may sorb or react with substances in the dilution water, allowing this equilibrium to become established before the test begins lessens the chances of changes in water chemistry occurring during at test. Even after extensive washing, new facilities still may contain toxic residues. The best way to determine if toxic residues remain is to test for their presence by maintaining or rearing the test fish species in the facility for a period of time equal to or exceeding the time required to complete a test.

f. Dilution Water

A constant supply of good quality dilution water is needed to maintain consistent experimental conditions during testing. A change in water quality during a test may alter the response of the test fish to the test solution. Most research on the effects of water quality have centered around the effects of changes in pH and total hardness on the acute and subacute toxicity of compounds. Mauck et al. (1977) performed static, acute tests with bluegill and Mexacarbate at various pH's. They observed that Mexacarbate was 38 times more toxic at a pH of 9.5 and 5 times more toxic at a pH of 8.5 than at a pH 7.5. They ascertained however that these large increases in toxicity were mostly caused by the rapid hydrolization of the parent compound to more toxic breakdown products, and not to an increase in sensitivity of the fish at the higher pH levels.

In another study of the effects of pH, Mauck et al. (1976) found that the acute toxicity of five pyrethroids did not change as the pH of the dilution water was increased from 7.5 to 9.5. The toxicity of pyrethrum extract however decreased as the LC50 increased from 41 mg/l at a pH of 6.5 to 87 mg/l at a pH of 9.5.

In a study with the formulated herbicide, Roundup®, Folmar et al. (1979) determined that the herbicide was five times more toxic to rainbow trout as the pH increased from 6.5 to 7.5. Additional increases to pH's of 8.5 and 9.5 did not further increase the toxicity. When bluegill were similarly tested, there was only a 2 fold increase in toxicity between a pH of 6.5 and 7.5.

When the toxicity of nitrite was tested at different pH levels, an inverse trend relationship was observed; toxicity decreased with increasing pH (Wedemeyer and Yasutake 1978). In static tests with steelhead trout (Salmo gairdneri) the toxicity decreased 8-fold for 5 g fish and 3-fold for 10g fish when the pH was increased from 6.0 to 8.0.

To estimate the potential chronic effects of reduced pH on freshwater fishes, chronic toxicity tests were performed with the fathead minnow by Mount (1973) and with the brook trout (Salvelinus fontinalis) by Menendez (1976). The results of both studies were similar; hatchability of eggs was reduced at pH levels <6.5. Both authors recommended that the pH of water should be above 6.5 to fully support the growth and reproduction of these fishes.

Much work has been performed studying the ameliorating effects of increased hardness on the toxicity of heavy metals to freshwater fish (Carrol et al. 1979, Holcombe and

Andrew 1978, Howarth and Sprague et al. 1976), but much less has been done with organic compounds. In the study by Mauck et al. (1977) the toxicity of Mexacarbate to brown trout (Salmo trutta) sac fry and Coho salmon (Oncorhynchus kisutch) fingerlings did not change when the hardness of the dilution water was increased from 40-48 mg/l to 160-180 mg/l.

Mauck et al. (1976) found only slight variations in LC50 values when they performed static, acute, toxicity tests with bluegill and five pyrethroids at hardnesses of 10-13, 40-48, 160-180, and 280-320 mg/l. The LC50 of pyrethrum was however significantly reduced from 62 to 46.5 mg/l as the hardness was increased from 10-13 to 280-320 mg/l. Wedemeyer and Yasutake (1978) found that the toxicity of nitrite to 5g steelhead trout decreased 24 times as the hardness was increased from 25 to 300 mg/l.

Although the reported data demonstrate that relatively large differences in the pH and hardness of the dilution water (> 2x) can effect the toxicity of a compound, it is not known what role,if any, small changes or even large gradual changes (> 2x) will have on the acute toxicity of compounds.

Brungs et al. (1976) performed a fathead minnow chronic toxicity test with copper using water collected downstream from a sewage treatment plant as the dilution water. Throughout testing, the hardness varied from 88-352 mg/l, akalinity from 50 to 248 mg/l, pH from 7.5 to 8.5, DO from 5.0 to 13.0 and temperature from 0 to 30°C. When results from this test were compared to the results of a similar chronic test performed with constant quality dilution water

(Mount 1978), it was shown that the variations in water quality had little or no effect on the toxicity of copper.

McLeay et al. (1979) performed static acute toxicity tests with rainbow trout and a pulp and paper mill effluent using 10 different dilution waters. Dilution water hardness ranged from 5 to 400 mg/l, pH from 6.4 to 8.4, conductivity from 15 to 778 umhos/cm, and alkalinity from 11 to 392 mg/l. The 24 hour LC50 values using the 10 waters ranged from 4.4 to 15.6% effluent and was pH related. After adjusting the pH of each dilution water to 6.5, the variation in the LC50's was reduced to a range of 4.4 to 6.9% effluent, indicating little effect due to the other measured and non-measured dilution water characteristics.

Mattson et al. (1976) performed static, acute toxicity tests with five organic compounds and fathead minnows in Lake Superior water and in reconstituted soft water and found no differences. Although no data on measured water quality paramaters of each dilution water were presented, the similarity of data from tests done in two obviously different dilution waters is noteworthy.

Although there is little data demonstrating that changes in the quality of the dilution water during testing will affect the test results, the dilution water should be kept as constant as possible during testing to minimize such a risk.

A dependable source of clean surface or well water usually will provide water having greater consistency in its chemical makeup than water from a municipal water supply. Municipal water may originate from several sources which differ in chemical makeup. In addition, municipal water

frequently is treated chemically as part of a purification process. Since the proportions in which water from different sources are mixed, and since the chemical treatment given water during the purification process may be different from time to time, the chemical makeup of municipal water may vary considerably. Reconstituted water, while theoretically more consistent from batch to batch than either surface or ground water or municipal water, may in some instances lack trace minerals required by some species of fish. Cairns (1969) performed many acute toxicity tests on some compounds with both reconstituted water and natural water and found that the data generated from the tests in natural water were not consistent or reproducible whereas the results from the tests with reconstituted water were consistent. Of more concern, however, is the prohibitive expense of continuously preparing reconstituted water for use in fish-holding and flow-through toxicity tests.

Fish culturists do not know all of the conditions required to maintain healthy fish, nor do they know all of the components and combination of components in water that adversely affect the health of fish (Brauhn and Schoettger 1975). Nevertheless, to avoid possible inconsistencies and inaccuracies in test results, healthy fish are needed for use in toxicity tests. There is, therefore, a need to determine that the dilution water, whatever its source, is able to support the fish species to be used in a healthy condition for the duration of the holding and testing periods.

An appropriate way to make that determination is to place young fish of a sensitive species, preferably the one

to be used in subsequent tests, in the dilution water for an extended period of time and observe their behavior, growth and development. Ideally, those observations should be made by an experienced fish culturist familiar with certain stress reactions which are difficult for an untrained observer to identify.

Surface and ground water may vary considerably in their chemistry depending upon the season of the year and precipitation patterns. Variations in the chemistry of surface water may involve the quantity of particulate matter, dissolved organic and inorganic chemicals, un-ionized ammonia, residual chlorine and various other contaminants. As an indication of uniformity of the dilution water used in the toxicity tests, it is recommended in the guideline that certain water chemistry parameters be measured at least twice a year, or more frequently if it is suspected that one or more of those parameters has changed significantly The water chemistry parameters singled out and the maximum acceptable concentrations listed for these parameters are among those generally accepted as substances and concentrations which do not adversely affect freshwater fish (APHA 1975, ASTM 1980). Recognizing that some variation in water chemistry is normal in natural surface waters, a 10 percent fluctuation from month to month in water hardness, akalinity, and conductivity, and a variance of 0.4 pH units is accepted as suitable.

g. Carriers

A carrier may be used to aid in the dissolution of a test compound into dilution water only after significant efforts to dissolve it in dilution water of dilution water

stocks have failed. Schoor (1975) believes that the use of a carrier may interfere with the uptake of the test compound by the test organisms; if the carrier molecules affect the adsorption of the test compound at the gill surface, there will be a resultant change in the rate of transport into the test organism. The author also states that the use of a carrier may increase the concentration of compound in the test solution above solubility by creating a stable water emulsion.

When a carrier is required, triethylene glycol (TEG), dimethyl formamide (DMF) or acetone may be used. The solvents should be tried in the order stated due to their relative toxicity to fathead minnows as reported by Cardwell et al. (manuscript 1980). The minimum amount should be used and the concentration of TEG should not exceed 80 mg/l, the MATC (maximum acceptable toxicant concentration) value. Concentrations of DMF and acetone should not exceed 5.0 mg/l, the MATC for DMF. Although there is no MATC value for acetone, its acute toxicity is similar to that of DMF.

Ethanol should not be used due to its tendancy to stimulate the excessive growth of bacteria in the test chambers.

4. Environmental Conditions

a. Loading

In the static tests, the loading should not be so high to deplete the dissolved oxygen or result in significant depletion of the toxicant due to uptake of the chemical by the fish. A maximum loading of 0.5 g/l will generally be sufficient for compounds that do not have a high bioconcentration potential, or are not likely to reduce the

dissolved oxygen concentration due to degradation. For other chemicals, flow-through tests should be performed.

A maximum loading rate of 0.5 g/l day flow-through tests should be sufficient to maintain proper dissolved oxygen and ammonia concentrations, and the proper concentration of the test chemical in the test solution.

In a flow-through study by Blanchard et al. (1977) a loading of 1.9 g/l day was not sufficient to prevent loss of 14 C-sec-butyl-4-chlorodiphenyloxide from the test water. The concentration of test substance decreased more than 50% during the first 12 hours of exposure and did not return to the expected concentration until after 72 hours.

In flow-through studies with 2 strains of rainbow trout, Alexander and Clarke (1978) tested phenol at three different loading rates of 0.7, 1.4, and 2.6 g/l day and found no significant differences in MST's between the three rates for each strain. These data indicate that at least for phenol loading up to 2.6 g/l day is not an important factor.

b. Dissolved Oxygen

The level of dissolved oxygen maintained in a test chamber can influence the sensitivity of test organisms to a test substance. Increased acute toxicity of hydrogen cyanide was observed in various fish species with the dissolved oxygen concentration was below 5 mg/l or approximatly 60% saturation at 25°C (Smith et al. 1978). Fathead minnow growth was inhibited at a dissolved oxygen concentration between 5.0 mg/l and 7.3 mg/l at a temperature range of 15-25°C, equivalent to approximately 65% saturation (Brungs 1971).

In order to provide a minimum margin of safety in acute toxicity tests, a dissolved oxygen concentration greater than 50% of saturation is recommended as a minimum.

c. Temperature

Since fish are poikilothermic, nearly all their biochemical processes are affected by the water temperature to which they are exposed. Prosser (1973) states that for every 10°C rise in temperature, the metabolism of fish normally increases by a factor of two. Therefore, it is likely that the toxic effects of chemicals can be temperature dependent.

During 96-hour tests with mercuric chloride and rainbow trout, MacLeod and Pessah (1973) noted that increased toxicity was directly related to an increase in temperature. Similar results were seen for the herbicide Roundup® (Folmar et al. 1979) and quinaldine sulfate (Marking and Olson 1975). It should be recognized, however, that not all chemicals exhibit a temperature related variance to acute toxicity (Smith and Heath 1979).

The optimal temperature at which acute toxicity tests were conducted has yet to be identified, but there are some temperatures which have undergone wide spread use and acceptance. In accordance with these practices the Agency recommends that acute toxicity tests with the fathead minnow and bluegill be performed at 22± 1°C and tests with rainbow trout be performed at 12° ± 1°C.

d. Light

Although light is recognized as a potentially important environmental variable, very few studies have been performed evaluating its potential effects. McLeay and Gordon (1978)

found no difference in the toxicity of a pulpmill effluent between tests performed with 8/16 and 16/8 hour light/dark photoperiods. To avoid any possible effects from extraneous light sources in the laboratory, the recommended photoperiod is 16-hours light, 8-hours dark, with a 15-30 minute transition period.

C. Reporting

A coherent theory of the concentration-response relationship was introduced by Bliss (1935), and is widely accepted today. This theory is based on four assumptions: (a) response is a positive function of dosage, i.e., it is expected that increasing treatment rates should produce increasing responses, (b) randomly selected animals are normally distributed with respect to their sensitivity to a toxicant, (c) due to homeostasis, response magnitudes are proportional to the logarithm of the dosage (stresses) to produce arithemtically increasing responses (strains) in test animals populations, (d) in the case of direct dosage of animals, their resistance to effects is proportional to body mass. Stated another way, the treatment needed to produce a given response is proportional to the size of the animals treated.

The concentration-response curve, in which percent mortality is plotted as a function of the logarithm of test substance concentration, can be interpreted as a cumulative distribution of tolerance within an experimental population (Hewlett and Plackett 1979). Experiments designed to measure tolerance directly (Bliss 1944) have shown that tolerance, in most cases, is lognormally distributed within a population. Departures from the lognormal pattern of

distribution are generally associated with mixtures of very susceptible and very resistant individuals (Hewlett and Plackett 1979). In addition, mixtures of toxicants can produce tolerance curves which deviate significantly from the lognormal pattern (Finney 1971).

If tolerance is lognormally distributed within the test population, the resulting concentration-response curve will be signmoidal in shape, resembling a logistic population curve (Hewlett and Plackett 1979). While estimates for the median lethal dose can be made directly from the concentration-response curves, a linear transformation often is possible, using probit (Bliss 1934, Finney 1971) or logit (Hewlett and Plackett 1979) transformations.

Once the mortality data have been transformed, a straight line can be fitted to the data points. Although this line is most often fitted by eye (APHA 1975), a least squares linear regression procedure is strongly recommended for this purpose (Steel and Torrie 1960). From the regression equation, confidence limits can be determined for predicted mortality values. An additional advantage is that the significance of the slope of the regression line can be determined (Draper and Smith 1966). By using replicate tests, and analysis of variance can be performed to determine whether deviations of data points from the regression line are random fluctuations or indications that a linear model is an inappropriate representation of the data points (Draper and Smith 1966).

While the values for the median lethal dose, LC50, can be estimated graphically from linearlized concentration-response curve, other techniques are preferable since the

graphical method does not permit the calculation of a confidence limit (APHA 1975). The probit method uses probit transformation and the minimum likelihood curve fitting technique (Finney 1971). The Litchfield and Wilcoxon method is a modified probit methods which does not require partial kills, as does the unmodified probit method (Litchfield and Wilcoxon 1949). The logit method utilizes either the maximum likelihood or the minimum Chi square method to estimate the LC50 (Ashton 1972, Berkson 1949). The moving average method is simple to apply but depends on the symmetry of the tolerance distribution to provide accurate estimates (Thompson 1947). It cannot be utilized to calculate any concentration level other than the LC50. An additional disadvantage is that confidence limits for the LC50 cannot be calculated if no partial kills are available.

The lack of partial kills seriously impairs the utility of the probit, logit, and moving average methods. In situations where there are no partial kills the binomial test (Siegel 1956) can be used to estimate the confidence limits around the LC50 value (Stephan 1977). The LC50 value can be calculated from the relation

$$LC50 = (A\ B)\ 1/2$$

Where

A = concentration at which no organisms die
B = concentration where all organisms die

A and B are the confidence limits of the estimate and

are significant above the 95 percent level if six or more tests organisms are exposed at each concentration level.

If dose-response data are plotted for each 24 hour interval throughout the test, the LC50 determined from each curve can be plotted as a function of time, yielding an acute toxicity curve (APHA 1975). This curve may approach the time axis asymptotically, indicating the final or threshold value of the LC50. The absence of a threshold LC50 may indicate the need for a test of longer duration.

The LC50 value has limited utility, since a number of substances with entirely different toxicity characteristics can produce identical LC50 numbers. The difference will therefore be in the slope of the concentration-response curve (Casarett and Doull 1975).

The majority of response data will produced a near-linear regression line. Yet very valuable information is gained when the regression line is found to deviate significantly from a straight line. For example, in fish bioassays, the concentration-response line can appear straight from the one percent to the 40 percent effect level and then bend abruptly to the horizontal. Above a certain level of test substance concentration no further mortality of fish occurs. Further increments of test substance simply precipitate from solution and become unavailable to fish. A low slope or broken regression line can occur when the experimenter has inadvertently mixed two populations of experimental animals (markedly different in their susceptibility) together at each treatment level.

III. Economic Aspects

The Agency awarded a contract to Enviro Control, Inc. to provide us with an estimate of the cost for performing static and flow-through acute toxicity tests according to this Guideline. Enviro Control supplied us with two estimates; a protocol estimate and a laboratory survey estimate.

The protocol estimate was $621 for a static test and $743 for a flow-through test. These estimates were prepared by separating the Guideline into individual tasks and estimating the hours to accomplish each task. Hourly rates were then applied to yield a total direct labor charge. An overhead rate of 115%, other direct costs of $40 for static and $50 for flow-through tests, a general and administrative rate of 10%, and a fee of 20% were then added to the direct labor charge to yield the final estimate.

Enviro Control estimated that differences in salaries, equipment, overhead costs and other factors between laboratories could result in as much as 50% variation from this estimate. Consequently they estimated that test costs could range from $310 to $931 for static tests and $372 to $1115 for flow-through tests

The laboratory survey estimate was $471 for static tests and $795 for flow-through tests. Five laboratories supplied estimates of their costs to perform the tests according to this Guideline. These costs ranged from $300 to $625 for static tests and $550 to $1250 for flow-through tests. The reported estimate is the mean value calculated from the individual costs.

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EG-10
August, 1982

FISH BIOCONCENTRATION TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

FISH BIOCONCENTRATION TEST

(a) Purpose. This guideline is intended to be used for assessing the propensity of chemical substances to bioconcentrate in freshwater fish. This guideline describes a bioconcentration test procedure for the continuous exposure of fathead minnows (Pimephales promelas) to a test substance in a flow-through system. The United States Environmental Protection Agency (EPA) will use data from this test in assessing the hazard a chemical may present to the environment.

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

(1) "Acclimation" is the physiological compensation by test organisms to new environmental conditions (e.g. temperature, hardness, pH).

(2) "Bioconcentration" is the net accumulation of a substance directly from water into and onto aquatic organisms.

(3) "Bioconcentration factor (BCF)" is the quotient of the concentration of a test substance in aquatic organisms at or over a discrete time period of exposure divided by the concentration

in the test water at or during the same time period.

(4) "Carrier" is a solvent used to dissolve a test substance prior to delivery of the test substance to the test chamber.

(5) "Depuration" is the elimination of a test substance from a test organism.

(6) "Depuration phase" is the portion of a bioconcentration test after the uptake phase during which the organisms are in flowing water to which no test substance is added.

(7) "Dilution water" is the water to which the test substance is added and in which the organisms undergo exposure.

(8) "Loading" is the ratio of fish biomass (grams, wet weight) to the volume (liters) of test solution passing through the test chamber during a 24-hr. period.

(9) "Organic chlorine" is the chlorine associated with all chlorine-containing compounds that elute just before lindane to just after mirex during gas chromotographic analysis using a halogen detector.

(10) "Organochlorine pesticides" are those pesticides which contain carbon and chlorine such as aldrin, DDD, DDE, DDT, dieldrin, endrin, and heptachlor.

(11) "Steady-state" is the time period during which the amounts of test substance being taken up and depurated by the test organisms are equal, i.e., equilibrium.

(12) "Steady-state bioconcentration factor" is the mean concentration of the test substance in test organisms during steady-state divided by the mean concentration in the test solution during the same period.

(13) "Stock solution" is the concentrated solution of the test substance which is dissolved and introduced into the dilution water.

(14) "Test chamber" is the container in which the test organisms are maintained during the test period.

(15) "Test solution" is dilution water containing the dissolved test substance to which test organisms are exposed.

(16) "Uptake" is the sorption of a test substance into a l onto aquatic organisms during exposure.

(17) "Uptake phase" is the initial portion of a bioconcentration test during which the organisms are exposed to the test solution.

(c) Test procedures--(1) Summary of the test. (i) Fathead minnows are continuously exposed to at least one constant sublethal concentration of a test substance under flow-through conditions for a maximum of 28 days. During this time, test solution and fish are periodically sampled and analyzed using

appropriate methods to quantify the test substance concentration. If prior to day 28, the tissue concentrations of the substance sampled over three consecutive sampling periods have been shown to be statistically similar (i.e., steady-state has been reached), the uptake phase of the test may be terminated and the remaining fish transferred to untreated flowing water until 95 percent of the accumulated residues have been eliminated, or for a maximum depuration period of 14 days.

(ii) The mean test substance concentration in the fish at steady-state is divided by the mean test solution concentration at the same time to estimate the bioconcentration factor (BCF).

(iii) If steady-state is not reached during 28 days of uptake, the steady-state BCF is calculated using non-linear parameter estimation methods.

(2) [Reserved]

(3) [Reserved]

(4) Definitive test--(i) Background information. The following data on the test substance should be known prior to testing:

(A) Its solubility in water.

(B) Its stability in water.

(C) Its octanol-water partition coefficient.

(D) Its acute toxicity to fathead minnows.

(E) The validity accuracy and minimum detection limits of the proposed analytical methods.

(ii) Selection of test concentration. (A) At least one concentration should be tested to assess the propensity of the compound to bioconcentrate. The concentration selected should not stress or adversely affect the fish and should be less than one-tenth the 96-hr or incipient LC50 determined from a flow-through test with fathead minnows. The test concentration should be less than the solubility limit of the compound in water and close to the potential or expected environmental concentration. The limiting factor of how low one can test is based on the detection limit of the analytical methods. The concentration of the test material in the test solution should be at least 3 times greater than the detection limit in water.

(B) If it is desired to document that the potential to bioconcentrate is independent of the test concentration, at least two concentrations should be tested that are at least a factor of 10 apart.

(iii) Estimation of test duration. (A) An estimate of the length of the uptake and depuration phases should be made prior to testing. This will allow the most effective sampling schedule to be determined. The uptake phase should continue until steady-state has been reached, but need not be longer than 28 days. The test should continue for at least 4 days.

(B) The time to steady state (S in hours) can be estimated from the water solubility or the octanol-water partition coefficient using the following equations:

$$S=3.0/\text{antilog}\ (0.431 \log W-2.11)\ \text{or,}$$

$$S=3.0/\text{antilog}\ (-0.414 \log P + 0.122)$$

where

W = water solubility (mg/l and

P = octanol-water partition coefficient

Based upon the estimate of the time to steady state, one of the following sampling schemes may be used to generate the appropriate data.

Time to Steady-State in Days

Test period	S<4	S>4<14	S>15<21	S>21
	Sampling Days			
Exposure	1[a]	4[a]	1	1
	6[a]	1	3	3
	1	3	7	7
	2	7	10	10
	3	10	14	14
	4	12	18	21
		14	22	28
Depuration	1[a]	1	1	1
	6[a]	2	3	3
	12[a]	4	7	7
	1	6	10	10
				14

a=hours

(C) The depuration phase should continue until at least 95 percent of the accumulated test substance and metabolites have been eliminated, but no longer than 14 days.

(iv) Test initiation. (A) The test should not be started until the test substance delivery system has been observed to be functioning properly for at least 48 hours. This time should be sufficient to allow the test substance concentration to become equilibrated with the test exposure system. Analyses of two sets of test solution samples taken prior to test initiation should document this equilibrium (i.e., the concentrations do not vary more than 20% from each other). At initiation (time 0), test solution samples should be collected immediately prior to the addition of fish to the test chambers.

(B) The appropriate number of fathead minnows should be impartially distributed to each test chamber up to five at a time until the appropriate numbers have been distributed. The exact number of test organisms depends upon the expected length of testing, sample size, and the number of additional specialized analyses to be performed at termination.

(v) Feeding. (A) Fish should be fed once a day throughout the uptake and depuration phases. Feeding should always be done just after sampling to minimize the effects of the test substance

present in the gut when sampling. Fish should be fed the same food at a similar quantity as they received during holding and acclimation.

(B) Uneaten food and fecal material should be removed from the test aquaria within 30 minutes after feeding to minimize uptake of test substance by the food or feces.

(vi) Observations. (A) Observations on fish appearance and behavior should be made and recorded daily. Any abnormal behavior such as erratic swimming, lethargy, increased excitability, or any changes in appearances or physiology such as discoloration, hyperventilation or opaque eyes should be recorded.

(B) Observations on compound solubility should also be recorded. These include the appearance of surface slicks, precipitates, or material adsorbing to the test chamber.

(vii) Water quality measurements. The water temperature and dissolved oxygen concentration should be recorded at least daily and the pH twice weekly in each test chamber during uptake and depuration.

(viii) Sampling procedures. (A) At each of the designated sampling times, triplicate water samples and enough fish should be collected from the exposure chamber(s) to allow for at least four fish tissue analyses. A similar number of control fish

should also be collected at each sample point, but only fish collected at the first sampling period and weekly thereafter should be analyzed. Triplicate control water samples will be collected at the time of test initiation and weekly thereafter. Test solution sam les should be removed from the approximate center of the water column.

(B) At each sampling period, the appropriate number of fish is netted and removed from each test chamber. Care should be taken not to sample the weakest and consequently usually the smallest fish, especially during the first few sampling periods, to prevent biasing the test results. Each fish is pithed, blotted dry and then frozen at <-10°C if not analyzed within four hours.

(C) At termination, an extra set of fish should be sampled and eviscerated for quantifying the residues in the viscera and carcass. If a radio-labelled test compound is used, a sufficient number of fish should be sampled at termination to permit identification and quantitation of any major (>10% of parent) metabolites present. It is crucial to determine how much of the activity present in the fish is directly attributable to the parent compound.

(5) Test results--(i) Biological. (A) The maximum allowable mortality of fish is 10 percent per week. If more than

10 percent of the fish in the control or test chamber(s) die during any week of testing, the test should be repeated.

(B) Steady-state has been reached when the mean concentrations of test substance in whole fish tissue taken on three consecutive sampling periods are statistically similar (F test, P=0.05). A BCF is then calculated by dividing the mean tissue residue concentration during steady-state by the mean test solution concentration during this same period. A 95 percent confidence interval should also be derived for the BCF. This can be done by calculating the mean fish tissue concentration at steady state (X_f) and its 97.5 percent confidence interval, $\pm$ t (S.E.), where t is the t statistic = 0.025 and S.E. is one standard error of the mean. This calculation would yield lower and upper confidence limits (L_f and U_f). The same procedure can be used to calculate the mean and 97.5 percent confidence interval from the test solution concentrations at steady-state, $X_s \pm t$ (S.E.), and the resulting upper and lower confidence limits (L_s and U_s). The 95 percent confidence interval of the BCF would then be between L_f/U_s and U_f/L_s.

(C) If steady-state was not reached during the 28 day uptake period, the maximum BCF should be calculated using the mean tissue concentration from that day and the mean water concentration from that and the previous sampling day. An uptake

rate constant should then be calculated using appropriate techniques, such as the BIOFAC program developed by Blau and Agin (1978). This rate constant will allow the estimation of a steady state BCF and the estimated time to steady-state.

(D) If 95 percent eliminati n has not been observed after 14 days depuration, then a depuration rate constant should be calculated. This rate constant will allow estimation of the time to 95% elimination.

(ii) Analytical. (A) All samples should be analyzed using EPA methods and guidelines whenever feasible. The specific methodology used should be validated before the test is initiated. The accuracy of the method should be measured by the method of known additions. This involves adding a known amount of the test substance to three water samples taken from an aquarium containing dilution water and a number of fish equal to that to be used in the test. The nominal concentration of these samples should be the same as the concentration to be used in the test. Samples taken on two separate days should be analyzed. The accuracy and precision of the analytical method should be checked using reference or split samples or suitable corroborative methods of analysis. The accuracy of standard solutions should be checked against other standard solutions whenever possible.

(B) An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences, unless it is shown that such degradation products are not present in the test c ambers during the test. Atomic absorption spectrophotometric methods for metals and gas chromatographic methods for organic compounds are preferable to colorimetric methods.

(C) In addition to analyzing samples of test solution, at least one reagent blank should also be analyzed when a reagent is used in the analysis.

(D) When radiolabelled test compounds are used, total radioactivity should be measured in all samples. At the end of the uptake phase, water and tissue samples should be analyzed using appropriate methodology to identify and estimate the amount of any major (≥ 10 percent of the parent compound) degradation products or metabolites that may be present.

(6) [Reserved]

(d) Test conditions--(1) Test species (i) Selection. (A) The fathead minnow (Pimephales promelas) should be used as the test organism.

(B) Immature fish should be used. They should be young enough so as not to mature during the test. Fish used in the same test should be as similar in size

as possible to reduce variability. The standard deviation of the weight should be less than 20 percent of the mean (N= 30).

(C) Fish used in the same test should be from the same supplier or culture unit and from the same holding and acclimation tank(s).

(D) Fathead minnows should not be used if they appear diseased or otherwise stressed or if more than 5 percent die during the 48 hours prior to testing. Diseased fish should be discarded or treated and held for a minimum of 14 days before testing.

(ii) Care and handling. (A) Fish purchased from a commercial source should be attended to immediately upon arrival. Transfer of the fish from the shipping to the holding water should be gradual to reduce stress caused by differences in water quality characteristics and temperature. Fish should be quarantined and observed for at least 14 days prior to testing.

(B) During holding, the fish should not be crowded and the dissolved oxygen concentration should be above 60 percent saturation. Holding tanks should be kept clean and free of debris. Fish should be fed at least once a day with a food which will support their survival and growth.

(C) Fish should be handled as little as possible. When handling is necessary, it should be done as gently, carefully,

and quickly as possible using dip nets made of small mesh nylon, silk, bolting cloth, plankton netting, or other similar knotless materials. Handling equipment should be sterilized between uses by autoclaving, treating with an iodophor or with 200 mg hypochlorite/liter.

(iii) Acclimation. If the holding water is not from the same source as the test dilution water, acclimation to the dilution water should be done gradually over a 48-hour peroid. The fish should then be held an additional 14 days in the dilution water prior to testing. Any changes in water temperature should not exceed 3°C per day. Fish should be held for a minimum of 7 days at the test temperature prior to testing.

(iv) Loading. The number of fish placed in each test chamber and the flow rate through the test chamber should be such that the uptake of the test substance by fish upon introduction into the test solution does not reduce the measured concentration of the test solution by more than 20 percent of the concentration measured before the fish were introduced. The loading should not exceed 0.1g fish per liter of test solution delivered over any 24 hour period, and the minimum turnover rate should be 6 aquaria volumes per 24 hours. For some compounds, loading rates less than 0.1g/l may be needed to prevent a substantial loss of test substance as a result of fish uptake.

(2) Facilities--(i) Dilution water. (A) A constant supply of good quality water should be available throughout the holding, acclimation and testing periods. Although unadulterated well water is recommended, de-chlorinated tap water or reconstituted soft water may be used. A dilution water is acceptable if fathead minnows will survive and grow normally for 60 days without exhibiting signs of stress, i.e., discoloration, lack of feeding, poor response to external stimuli, or lethargy.

(B) The total hardness, alkalinity, pH, specific conductance, temperature and dissolved oxygen concentration of the dilution water should be determined weekly. The pH should not vary more 0.4 units and the other parameters more than 10 percent on a monthly basis.

(C) Reconstituted soft water, if used, should be prepared by adding 4.8 g $NaHCO_3$, 3.0 g $CaSO_4$ $2H_20$, 3.0 g $MgSO_4$, and 200 mg KCl to each 100 l of deionized or glass distilled water, or to dechlorinated tap water with a total residual chlorine concentration less than 1 ug/l. In all cases the specific conductance at 25°C of the water source should be less than 1 micromho/cm.

(D) All water should be extensively aerated prior to use if the dissolved oxygen concentration is less than 90 percent of

saturation. If the concentration of dissolved gases exceeds 110 percent of saturation, the excess gases should be removed using appropriate apparatus.

(E) The quality of the dilution water should be constant and should meet the following specifications measured at least twice a year.

Substance	Maximum Concentration
Particulate matter	20 mg/liter
Total organic carbon	2 mg/liter
or	
Chemical oxygen demand	5 mg/liter
Un-ionized ammonia	1 ug/liter
Residual chlorine	1 ug/liter
Total organophosphorus pesticides	50 ng/liter
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs)	50 ng/liter
or	
Organic chlorine	25 ng/liter
Copper, cadmium or zinc	10 ug/liter

(ii) Construction materials. Materials and equipment that contact dilution water, stock solutions or test solutions should not leach or absorb substances. Glass, #316 stainless steel and perfluorocarbon plastics (e.g. Teflon ®) should be used whenever possible. Concrete, unplasticized plastics and fiberglass may be used for holding and acclimation tanks and in the water supply system, but they should be thoroughly conditioned before use by rising with a continuous flow of water > 25°C for 48 hours. The use of flexible tubing should be avoided as phthalate esters leach from these materials. Cast iron pipe may be used but filters will be needed to remove rust particles. Rubber, copper, brass, galvanized metal, and epoxy glue should not come in contact with dilution water, stock solutions or test solutions.

(iii) Fish holding and acclimation. (A) Tanks are needed for holding and acclimating fathead minnows prior to testing. The number and size of tanks needed depends upon the amount of testing to be performed and the availability of fish of the right age. A constant supply of good quality dilution water should be supplied to all tanks. The volume required depends upon the holding temperature and the number of fish being held, but the flow should be great enough to maintain a dissolved oxygen concentration > 60 percent of saturation.

(B) Temperature control apparatus are needed to maintain the desired holding and acclimation temperatures. Apparatus controls should be able to maintain temperatures within 1°C of the appropriate temperature. If the water is heated, care should be taken to avoid supersaturation of gases in the water.

(iv) Testing apparatus. (A) Test chambers can be made from welded #316 stainless steel or from double strength glass joined with clear silicone adhesive. The size, shape and depth of the test chambers are not important as long as they accommodate the loading requirements.

(B) The test substance delivery system used should accommodate the physical and chemical properties of the test substance and the selected exposure concentration. The apparatus used should accurately and precisely deliver the appropriate amount of stock solution and dilution water to the test chambers. The introduction of the test substance should be done in such a way as to maximize the homogeneous distribution of the test substance throughout the test chamber.

(C) The dilution water should be delivered to an elevated headbox from which it can flow by gravity to the test substance delivery system. Use of a headbox facilitates a constant delivery rate and heating or cooling of the water to the approximate test temperature prior to delivery. Water in the

headbox may also be easily aerated or degassed as the situation dictates.

(v) Cleaning of test apparatus. Delivery systems and test chambers should be cleaned before and after each use. If there is obvious absorption of a test substance by the silicone adhesive, those applicable parts of the delivery system should be discarded.

(3) Test parameters--(i) Dissolved oxygen. The dissolved oxygen concentration in each chamber should be greater than 5.3 mg/l (60 percent of sea-level saturation at 22°C) throughout testing.

(ii) Temperature. The test temperature should be 22 ±1°C. Temporary excursions (< 8 hours) to 20 or 24°C are permissible.

(iii) Lighting. A photoperiod of 12 hours light and 12 hours dark with a 15-30 minute transition period is recommended.

(iv) Test substance. The name and purity of the test substance to be tested will be specified in the test rule. Radio-labelled compounds should not be used unless there are no suitable, validated, analytical techniques to measure unlabelled test substance in fish, or the costs of these analytical techniques are very high.

(v) Carrier use. Whenever possible, the test substance should be added directly to the dilution water or from a water

stock solution. With compounds having a low water solubility, it may be necessary to prepare test solutions using a carrier. The carriers to be used, in order of preference are: triethylene glycol (TEG), dimethyl formamide (DMF) and acetone. The amount used should be kept to a minimum and should not exceed 80 mg/l in the test solution for TEG and 5.0 mg/l for DMF and acetone.

(e) Reporting. In addition to the information required in Part 792--Good Laboratory Pracice Standards, the report should contain the following:

(1) The source of the dilution water, its mean monthly chemical characteristics (total hardness, alkalinity, pH, specific conductance, temperature and D.O.) and a description of any pretreatment.

(2) Detailed information about the fathead minnows used, including age, mean and standard deviation wet weight (blotted dry) and standard length, source, history of disease, parasites and treatment, acclimation procedures, and food used.

(3) The number of organisms tested, loading rate and volume additions per 24 hours.

(4) The percentage mortality of control fish and fish in each exposure chamber and any observed abnormal behavioral or physiological effects.

(5) The method of stock solution preparation including

nominal and measured concentrations and solvent used.

(6) The mean, standard deviation and range of the temperature, dissolved oxygen concentration and pH during the test period.

(7) Photoperiod length and light intensity.

(8) Description of sampling and analytical methods for water and tissue analyses.

(9) The mean, standard deviation and range of the concentration of test compound in the test solution and fish tissue at each sampling period.

(10) The time to steady-state.

(11) The steady-state or maximum BCF and the 95% confidence limits.

(12) The time to 95 percent elimination of accumulated residues.

(f) References. Blau GE , Agin CL. A users manual for BIOFAC: A computer program for characterizing the ratio of uptake and clearance of chemicals in aquatic organisms. Dow Chemical Co. March 15, 1978.

ES-7
August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

FISH BIOCONCENTRATION TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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Office of Toxic Substances ES-7
August, 1982

Technical Support Document for Fish Bioconcentration Test

I. Purpose

The purpose of this support document is to provide the scientific background and rationale used in the development of Test Guideline EG-10 to evaluate the bioconcentration potential of chemical substances in fish. The Document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures and conditions prescribed in the Test Guideline. Technical issues and practical considerations relevant to the Test Guideline are discussed. In addition, estimates of the cost of conducting the test are provided.

II. Scientific Aspects

A. Test Procedures

1. General

Fish are nearly unbiquitous inhabitants of freshwater and marine environments. In addition to their economic and recreational value, fish occupy on essential position in aquatic food chains, feeding on various forms of plant and animal life in the aquatic environment and, in turn, being eaten by some other aquatic or terrestrial consumer. Through these trophic or feeding interactions, nutrient and energy exchanges occur which are needed to maintain the ecological stability of the aquatic environment and sustain the food chains of commercially and recreationally valuable fish. Because they are critical links in these food chains, certain species of fish which have no direct commercial or recreational value in themselves are essential to the well-being of economically important fisheries. A chemical which

is highly cumulative could destroy an economically or ecologically important fish population through secondary toxicity or through contamination via food chain transfer or magnification (e.g. Kepone, PCB.).

In view of the economic and ecological importance of fish, and since man-made chemicals may be released directly or transported into aquatic environments, the tendency of some of those chemicals to accumulate in fish is of concern to us as we assess the risk they may pose to the environment. For this reason, reliable and adequate data on the bioconcentration potential of chemicals in fish should be available when the effects of those chemicals on the environment are assessed.

Data from a fish bioconcentration test can be used in conjunction with data on the acute toxicity and on the transport and fate of the chemical in an aquatic habitat, in assessing the risk resulting from the release of that chemical into the environment.

Although water solubility and octanol-water partitioning data have been shown to be useful tools in predicting bioconcentration factors (BCF), they provide reasonable estimates only within one order of magnitude of the degree to which most compounds may accumulate in fish tissue (Kenaga and Goring 1980, Neely et al. 1974, Veith et al. 1979, 1980). This information is also not useful for some heavy metals and inorganics that might bioconcentrate in non-lipid tissues such as bone. For some compounds such as hexachlorocyclopentadiene and chlorinated ecosane, however, the partition coefficient grossly overestimates the BCF (Veith et al. 1979). Such data, if used exclusively, could

result in over-regulation of compounds.

If data on water solubility, octanol-water partitioning, biodegradability, or structure-activity relationships suggest that a compound may substantially bioconcentrate in fish, a bioconcentration test with fathead minnows should be performed to quantitate empirically the degree to which such a compound may be accumulated in fish tissue.

There are two methodologies used today to estimate bioconcentration potential; the kinetic approach and the steady-state approach. Bishop and Maki (1980) and Hamelink (1977) review both. Using the kinetic approach, Bishop and Maki(1980), Branson et al. (1975), Cember et al. (1978) and Krzeminsky (1977) proposed the use of first-order kinetic expressions from relatively short ($\leq$ 5 days) fish exposure and depuration periods to calculate uptake and depuration rate constants. These rate constants are then used to estimate the BCF at the time of apparent steady-state, and the time to 50% elimination. The steady-state method, in more wide-spread use, exposes fish for a longer period of time until steady-state in the tissue is experimentally observed (Barrows et al. 1980, Bishop and Maki 1980, Veith et al. 1979) and continues with a depuration phase until 50 or 95% elimination has been observed. The estimation of bioconcentration using the kinetic approach can not account and adjust for changes in the rates of uptake and depuration such as those observed by Barrows et al. (1980) and Melancon and Lech (1979). The use of the kinetic approach also requires access to a sophisticated computer system, apparatus not readily available to many laboratories.

Although Bishop and Maki (1980) and Branson et al. (1975) have shown excellent agreement between estimates of bioconcentration factors for some compounds using both approaches, we recommend a modified steady-state method for determination of bioconcentration potential. The empirical nature of the data, the relative ease with which the test can be performed, and the number of researchers and laboratories that have performed such tests make this test more appropriate at this time. As the data base for comparisons of BCF's between the two methods grows, the kinetic approach may become more useful and valuable. Under the Toxic Substances Control Act, we are required to review all test guidelines annually, and in the future we will consider adopting the kinetic approach.

2. Test Substance Concentrations

Although virtually all researchers involved in bioconcentration testing state that the exposure concentration should be below toxic effect levels, there are few data supporting this recommendation. Tests determining bioconcentration factors with fathead minnows and PCBs (DeFoe et al. 1978), toxaphene (Mayer et al. 1977), three chlorinated cyclodiene intermediates (Spehar et al. 1979), and acrolein (Macek et al. 1976) showed that there was little difference between BCF's calculated from different exposure concentrations up to and including at least one concentration that caused a reduction in survival or growth.

Mayer (1976) however performed a study with di-2-ethylhexyl phthalate and fathead minnows and found that the BCF's increased sixfold, from 155 to 886, as the exposure concentration decreased from 62 to 1.9 ug/l. Bishop and

Maki (1980) tested four compounds with bluegills at two concentrations a factor of 10 apart. In the tests with DDT, tetradecylheptaethoxylate (AE), and EDTA, similar BCF's were observed at both concentrations tested. Elimination of accumulated residues however was considerably slower at the lower EDTA concentration. In the test with LAS however, the BCF at the low concentration of 0.064 mg/l was 260, more than twice the BCF observed at 0.63 mg/l. Of 15 compounds tested with bluegill at two concentrations by Macek et al. (1975), four yielded BCF's at the lower concentration that were 2.2 to 6.2 times the BCF at the higher exposure level. For two other compounds, the reverse was true; the BCFs at the higher concentration were 2.8 and 5.5 times those at the lower level. As even these differences were not great, available data does not warrant required testing of all compounds at two exposure concentrations.

What would be most useful for the hazard and risk assessment processes would be the use of an exposure concentration that approximates the expected or estimated environmental concentration. One should take care, however, that the selected concentration is at least three times its detection limit in water and will allow quantification of the residues in tissue. Test concentrations of 1-10 ug/l would be appropriate for many compounds.

3. Duration of Test

The exposure period should be long enough to demonstrate that steady-state has been reached in the fathead minnow tissue. Most compounds will reach steady state within the recommended 28 day maximum uptake period (Barrows et al. 1980, Bishop and Maki 1980, Macek et al. 1975, Veith et al.

1979). The depuration period should continue until at least 95% of the accumulated residues have been eliminated. This will normally occur within the 14 day maximum depuration period.

Most of the 33 chemicals tested by Barrows et al. (1980), reached steady-state in 3-10 days, and 50% depuration was usually reached in less than 1 day. Consequently it is clear that the relatively long uptake and depuration periods ($\geq$ 28 days uptake, 14 days depuration) used by many researchers are usually not required.

Before starting a bioconcentration test, an estimation of the BCF and the time to steady-state should be made. Kenaga and Goring (1980) present data and methods to estimate the BCF. The two most commonly used factors for predicting bioconcentration potential are water solubility and octanol-water partitioning. Water solubility can be determined empirically in the laboratory or, in some cases, taken from the literature (Chiou et al. 1977, Kenaga and Goring 1980). Octanol-water partition coefficients can be determined empirically, estimated by reverse-phase high pressure liquid chromatography according to Veith and Morris (1978), calculated according to Leo et al. (1971) or taken from the literature (Chiou et al. 1977, Hansch et al. 1972, Kenaga and Goring 1980).

The time to steady-state (S in hours) can be estimated from the water solubility or the octanol-water partition coefficient using the equations developed by ASTM (1980b):

S=3.0/antilog (0.431 log W-2.11) or

S=3.0/antilog (-0.414 log P + 0.122)

where

W=water solubility (mg/l) and

P=octanol-water partition coefficient

Presented below is a summary of data correlating various exposure times to the corresponding estimates of partition and BFC

S	P	Log P	Log BCF[a]	BCF
2	1,585	3.2	2.02	105
4	8,710	3.94	2.65	446
7	33,113	4.52	3.14	1 387
12	120,226	5.08	4.62	4150
18	316,228	5.5	4.0	10,000
22	524,807	5.72	4.16	14,521
28	933,254	5.97	4.37	23,686

[a]Log BCF was estimated using the equation of Veith et al. (1979) where log BCF=0.85 log P - 0.70.

Based on the estimate of the time to steady state, one of the following sampling schemes may be used to generate appropriate data.

Time to Steady-State

Test Period/	S[a]<4	S4-14	> S15-21	S>21
	Sampling Days			
Exposure	1[b]	4[b]	1	1
	6[b]	1	3	3
	1	3	7	7
	2	7	10	10
	3	10	14	14
	4	12	18	21
		14	22	28
Depuration	1[b]	1	1	1
	6[b]	2	3	3
	12[b]	4	7	7
	1	6	10	10
				14

[a]length of estimated time to steady state in days.
[b]hours.

B. Test Conditions

1. Test Species

a. Selection

The most common fish species used to determine the bioconcentration potential of compounds under flow-through conditions have been the fathead minnow, Pimephales promelas (Mayer 1976, Spehar et al. 1979, Veith et al. 1979); rainbow trout, Salmo gairdneri (Blanchard et al. 1977, Branson et al. 1975, Melancon and Lech 1979, Neely et al. 1974, Reinert et al. 1974); and bluegill Lepomis macrochirus (Barrows et al. 1980, Gonz et al. 1975, Macek et al. 1975).

The fathead minnow has been selected as the test species for use. It can be easily cultured in the laboratory (U.S. EPA 1971), thus insuring an almost constant supply of healthy fish of the proper size throughout the year. It has

been used extensively in life-cycle chronic toxicity tests and early-life stage tests as summarized by Macek and Sleight (1977) and McKim (1977). A definitive study on measuring and estimating the bioconcentration factor of chemicals in fish has also been performed using the fathead minnow as the test species (Veith et al. 1979). Results of this study clearly demonstrate the suitability of this species. In tests with hexachlorobenzene and 1,2,4-trichlorobenzene, the authors found that fathead minnows accumulated these compounds to the same extent as green sunfish (Lepomis cyanellus) and approximately twice as much as rainbow trout.

In a separate set of tests with hexachlorobenzene, the authors determined that the age and size of fathead minnows had little effect on bioconcentration. Tests with newly hatched fry, 30 and 90-day old juveniles, and approximately 180-day old adults yielded similar BCF's.

The source of the test fish was also found by Veith et al. not to be a significant source of variation in the bioconcentration of a PCB mixture (Aroclor 1016®). Tests with three different fish populations from a laboratory brood culture and with two populations from ponds yielded similar BCF's.

Studies by DeFoe et al. (1978) and Nebeker et al. (1974) demonstrated that gravid fathead minnows bioconcentrated PCB mixtures twice as much as males during laboratory tests. This increase was due to the increased lipid content of the females compared to the males. Consequently we recommend that immature fathead minnows less than 120 days old be used. Fish older than 120 days should not be used as by the

end of testing they may be sexually mature. The detection limits of the analytical procedures used to measure tissue residues may preclude the use of very small fish (eg. <30 days old) with compounds that do not bioconcentrate appreciably.

2. Maintenance of Test Species

a. Acclimation

Brauhn and Schoettger (1975) found that fish that had become accustomed to unrestricted swimming in rearing ponds underwent intense competition for food and swimming space when placed in confined holding tanks. These authors recommended that fish be maintained in confined holding tanks with color backgrounds and light intensities similar to those in the testing area to prevent additional stress when transferred to test chambers.

Although the importance of acclimation to the test temperature is still unclear, a maximum gradual change of 3°C/day is recommended at this time. Peterson and Anderson (1969) concluded that complete acclimation to temperature, based on changes in locomotor activity and oxygen consumption, requires approximately two weeks before metabolism is back to normal. They also determined that the rate of change was more important than the amount of change.

3. Facilities

a. Dilution Water

A constant supply of good quality dilution water is needed to maintain consistent experimental conditions during testing. A change in water quality during a test may alter the response of the test fish to the test solution. Although there is substantial information on the effects of

pH (Folmar et al. 1979, Mauck et al. 1976, 1977, Menendez 1976, Mount 1973) and hardness (Carroll et al. 1979, Holcombe and Andrew 1978, Howarth and Sprague 1978, Sauter et al. 1976) on the acute and chronic toxicity of compounds, there is no apparent data describing the effects of these water quality parameters on bioconcentration potential.

A dependable source of clean surface or ground water usually will provide water having greater consistency in its chemical makeup than that from a municipal water supply. Municipal water may have originated from several sources which differ in chemical makeup. Municipal water frequently is also treated chemically as part of a purification process. Since the proportions in which waters from different sources may be mixed, and since the chemical treatment given water during the purification process may be different from time to time, the chemical makeup of municipal water may vary considerably. Reconstituted water, while theoretically more consistent from batch to batch than either surface or ground water or municipal water, may in some instances lack trace minerals required by some species of fish. Cairns (1969), however, performed several acute toxicity tests on some compounds with both reconstituted water and natural water and found that the data generated from the tests in natural water were not consistent or reproducible, whereas the results from the tests with reconstituted water were consistent.

Fish culturists do not know all of the conditions required to maintain fish health, nor do they know all of the components and combinations of components in water that adversely affect the health of fish (Brauhn and Schoettger

1975). Nevertheless, to avoid possible inconsistencies and inaccuracies in test results, healthy fish are needed for use in bioconcentration tests. There is, therefore, a need to determine that the dilution water, whatever its source, is able to support the fathead minnow in a healthy condition for the duration of the test period.

An appropriate way to make that determination is to place young fathead minnows in the dilution water under flow-through conditions for 60 days and observe their behavior, growth and development. Ideally, those observations should be made by an experienced biologist familiar with certain stress reactions which are difficult for an untrained observer to identify (Brauhn and Schoettger 1975).

Surface and ground water may vary considerably in their chemistry depending upon the season of the year and precipitation patterns. Variations in the chemistry of surface water may involve the quantity of particulate matter, dissolved organic and inorganic chemicals, un-ionized ammonia and various other contaminants. As an indication of the uniformity of the dilution water, it is recommended in the guideline that certain substances be quantified at least twice a year or more frequently if it is suspected that the concentration of one or more of those substances have changed significantly. The maximum acceptable concentrations listed for these substances are among those generally accepted as concentrations which do not adversely affect freshwater fish (APHA 1975, ASTM 1980a). Concentrations in excess of the values cited in the guideline may affect the data developed from the

bioconcentration test.

Recognizing that some variation in water chemistry is normal in natural surface or ground waters, a 20 percent fluctuation from month to month in water hardness, alkalinity and conductivity and a variance of 0.4 pH units is acceptable.

b. Construction materials

Due to the toxicity of many heavy metals at low concentrations (U.S. EPA 1976) and the tendency of metal pipe, galvanized sheeting, laboratory equipment, etc. to leach metals into water, no metal other than stainless steel (preferably #316) should be used. For the same reason, plasticized plastics should not be used due to the high toxicity of the main component, di-2-ethyl-hexyl phthalate, (Mayer and Sanders 1973) which may leach into aquaria systems (Carmignani and Bennett 1976). To avoid any possible stress from exposure to low levels of metals, phthalate esters, and other potential contaminants, #316 stainless steel, glass and perfluorocarbon plastics (e.g. Teflon®) should be used whenever possible and economically feasible. If other plastics should be used, conditioning with a continuous flow of dilution water > 25°C should be performed for a minimum of 48 hours.

c. Testing apparatus

The size and shape of the test chambers are not important as long as they accomodate the loading requirements. The chambers should however, be large enough and contain enough water such that the fish are not stressed by crowding.

The following criteria presented by Hodson (1979) should be considered when selecting or designing a toxicant

delivery system: 1) capable of stopping the delivery of the toxicant if delivery of the dilution water stops, 2) consistent in delivery amounts throughout the test period, 3) independent of electrical failure, 4) independent of temperature and humidity fluctuations, 5) capable of delivering small quantities, 6) easy to construct with few moving parts and 7) easy to operate.

Any one of several types of toxicant delivery devices can be used as long as it has been shown to be accurate and reliable throughout the testing period. The greater the variation in the quantity of test substance introduced, the greater the spread of response values measured during testing. Syringe injector systems (Barrows et al. 1980, Spehar et al. 1979), metering pump systems (Veith et al. 1979) and modified proportional diluters (Macek et al. 1975, Neely et al. 1974) have been used successfully.

The solubility of the test compound should also be taken into account when selecting a delivery system. If the compound can be dissolved in water, a device capable of delivering amounts of test solution greater than 1 ml will probably be needed. If a carrier should be used, a system capable of accurately delivering very small amounts (< 100 ul) will be required to minimize the carrier concentration in the test solution.

d. Cleaning

Before use, the test system should be cleaned to remove dust, dirt and other debris and residue that may remain from the previous use of the system. Any of these substances may affect the results of a test by sorption of test materials or by exerting an adverse effect on test organisms. If any

test chambers or parts of the testing apparatus have obviously absorbed test compound, those chambers or parts should be discarded. New chambers should also be cleaned to remove any chemical or dirt residues that have accumulated during construction or storage. Detergent is used to remove hydrophobic or lipid-like substances. Acetone is used for the same purpose and to remove any detergent residues. It is important to use pesticide-free acetone to prevent the contamination of the chambers with pesticides which may be toxic to the test organisms or which might otherwise influence the outcome of the test. Nitric acid is used to clean metal residues from the system. The final rinse with water washes away the nitric acid. At the end of a test, the system should be washed in preparation for the next test.

Conditioning the test system with dilution water before it is used allows an equilibrium to be established between the chemicals in the water and the materials of the test system. Since a test system may sorb or react with substances in the dilution water, allowing this equibibrium to become established before the test begins lessens the chances of additional changes in water chemistry occurring during a test.

Even after extensive washing, new facilities still may contain toxic residues. A good way to determine if toxic residues remain is to test for their presence by maintaining fathead minnows in those facilities for a period of time equal to or exceeding the time required to complete a test.

e. Carriers

Carriers may be used to aid in the dissolution of test

compounds into dilution water only after significant efforts to dissolve it in dilution water stocks or test solutions have failed. Schoor (1975) believes that the use of a carrier may interfere with the uptake of the test compound by the test organism; if the carrier molecules affect the adsorption of the test compound at the gill surface, there will be a resultant change in the rate of transport into the test organism. The author also states that the use of a carrier may increase the concentration of a compound in the test solution above solubility by creating a stable water emulsion.

When a carrier is necessary, triethylene glycol (TEG), dimethyl formamide (DMF) or acetone may be used. The solvents should be tried in the order stated due to their relative toxicity to fathead minnows as reported by Cardwell et al. (manuscript, 1980). The minimum amount should be used, and the concentration of TEG should not exceed 80 mg/l, the MATC (maximum acceptable toxicant concentration) value. Concentrations of DMF and acetone should not exceed 5.0 mg/l, the MATC for DMF. Although there is no MATC value for acetone, its acute toxicity is similar to that of DMF.

4. Environmental Conditions

a. Loading

The flow rate through a test chamber should be high enough to maintain the dissolved oxygen concentration greater than 60% of saturation (5.3 mg/l at sea level and 22°C), minimize buildup of ammonia, and limit to 30% the loss of compound by adsorption onto the walls of the test apparatus and by absorption by the fish.

Different researchers have used widely different flow and loading rates. Analysis of the data reported by Veith et al. (1979) indicated that they used a loading rate of approximately 0.1 g/l/day and a turnover rate of 12. Spehar et al. (1979) used a loading rate of 0.125 g/l/day and had 27 aquaria turnovers per day. Barrows et al. (1980) used a loading rate that ranged from 0.4 to 1.0 g/l/day and a considerably slower turnover rate of 6-7. With many of the compounds studied by Barrows however, the compound concentration in water dropped substantially below what it was before the fish were introduced and usually took 1 to 3 days to recover to pre-test levels (Personnel communication, Barrows). In a study by Blanchard et al. (1977) a loading of 1.5 g/l/day and a turnover rate of 6 were not sufficient to prevent loss of 14C-sec-butyl-4-chlorodiphenyloxide from the test water. The concentration of the test substance decreased more than 50% during the first 12 hours of exposure and did not return to the expected concentration until after 72 hours. Such a phenomenon did not occur in the study by Veith (personal communication) where a higher flow rate was used.

We recommend a maximum loading rate of 0.1 g/l/day and a minimum turnover rate of 6. An even lower loading may be needed if the compound is suspected to readily degrade, is highly volatile or is expected to accumulate quickly and substantially in fish.

b. Dissolved Oxygen (See Loading)

c. Temperature

Since fish are poikilothermic, most biochemical activities are affected by the water temperature to which

they are exposed. Although there are some exceptions, Prosser (1973) states that there is approximately a 2 fold increase in fish metabolism for each 10°C rise in water temperature.

During 96-hour studies with rainbow trout and methyl mercuric chloride HgCl, MacLeod and Pessah (1973) found that accumulation of mercury increased with temperature and that this increase was due to an increase in metabolic rate.

Reinert et al. (1974) exposed rainbow trout to HgCl and DDT separately, at 5, 10 and 15°C and found that accumulation of mercury increased 78% between 5 and 15°C, and DDT accumulation increased 140% over the same temperature range. They stated that this increase was not due to the intrinsic nature of the chemicals but due to the increased metabolism of the fish.

In the study by Veith et al. (1979), fathead minnows were exposed to Aroclor 1254® at 5, 10, 15, 20 and 25°C. The log BCF's increased substantially between 5, 10 and 15°C and slightly between 20 and 25°C. There was little difference between log BCF's at 15 and 20°C.

Although researchers have performed apparently successful tests at 16°C (Barrows et al. 1980), 20°C (Macek et al. 1975) and 25°C (Veith et al. 1979), and there are some indications that greater BCF's will be generated at increased temperatures, we recommend testing at 22 ± 1°C. As testing at 25°C may induce sexual maturation (U.S. EPA 1971), the test temperature should be less than 25°C. A test temperature of 22°C is also consistent with the test temperature recommended in a similar TSCA test guideline for acute toxicity tests with fathead minnows. Having identical

test temperatures will limit the number of separate fish populations required to be held and acclimated at the testing laboratory.

d. Light

Although many researchers have used a 16 hour light - 8 hour dark photoperiod (Neely et al 1974, Spehar et al. 1979, Veith et al. 1979) and an ASTM task group has recommended its use in Draft 10 of the Proposed Practice for Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Molluscs (1980b), there is no scientific justification given for its use.

To retard gamete maturation, a photoperiod of 12 hours light-12 hours dark with a 15-30 minute transition period is recommended.

C. Reporting

An estimate of the time to steady state, the steady-state BCF, and the time to 50% or 95% elimination should be made for each compound tested. If steady-state has not been observed during the maximum 28 day exposure period or if 95% elimination has not been achieve during 14 days depuration, data generated during these tests should be used to estimate these values. The BIOFAC program developed by Blau and Agin (1978) uses nonlinear regression techniques to estimate the uptake and depuration rate constant, the steady-state BCF, the time to reach 90% of steady-state, the time to reach 50% elimination, and the variability associated with each estimate.

III. Economic Aspects

The Agency awarded a contract to Enviro Control, Inc. to provide us with an estimate of the cost for

performing a bioconcentration test using the fathead minnow according to this Guideline. Enviro Control supplied us with two estimates; a protocol estimate and a laboratory survey estimate.

The protocol estimate was $8,274. This estimate was prepared by identifying the major tasks needed to do a test and estimating the hours to accomplish each task. Appropriate hourly rates were then applied to yield a total direct labor charge. An estimated average overhead rate of 115%, other direct costs of $300, a general and administrative rate of 10%, and a fee of 20% were then added to the direct labor charge to yield the final estimate.

Enviro Control estimated that differences in salaries, equipment, overhead costs and other factors between laboratories could result in as much as 50% variation from this estimate. Consequently, they estimated that test costs could range from $4,137 to $12,411.

The laboratory survey estimate was $10,938, the mean of the estimates received from four laboratories. The estimates ranged from $6,000 to $15,750 and were based on the costs to perform the test according to this Guideline. Enviro Control listed the following as possible sources of variation in the cost estimates:

- o understanding the Guideline
- o overhead rates
- o salary rates
- o in-house expertise
- o worker productivity and efficiency
- o degree of automation
- o accuracy of protocol costing procedures

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16. Abstract (Limit: 200 words)

These documents consitute a set of 21 environmental effects test guidelines (and, in some cases, support documents) that may be cited as methodologies to be used in chemical specific test rules promulgated under Section 4(a) of the Toxic Substances Control Act (TSCA). These guidelines cover testing for invertebrate toxicity, aquatic vertebrate toxicity, avian toxicity, phytotoxicity, and bioconcentration. The guidelines will be published in loose leaf form and updates will be made available as changes are dictated by experience and/or advances in the state-of-the-art.

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