

PART 8000

TOXICITY

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SUMMARY OF MAJOR CHANGES SINCE 1998

Comet Assay (8071) is a new test procedure that measures the increased incidence of DNA damage resulting from contaminant exposure. Marine Macroalgae (8113) is a new test, for use on the Pacific Coast of North America, that relates percentage of germinated zoospores and growth of the germ tube to toxicant concentration. Amphibians (8930) is a new procedure describing the Frog Embryo Teratogenesis Assay-*Xenopus* Test (FETAX), which measures the effect of toxicants on the developmental processes in amphibians.

Mutagenesis (8030) includes updated experimental test procedures measuring microsomal mutagenicity (Ames test). Detailed procedures were added to Bacterial Bioluminescence (8050). P450 Reporter Gene Response (8070) contains additional details on conducting the test. A section on field testing of marine and freshwater pelecypods to measure survival, growth, and bioaccumulation was added to Mollusks (8610). The test protocol for the American lobster was added to Decapods (8740). Figures of test organisms and tables listing test requirements were added to many of the test procedures.

8010 INTRODUCTION*

8010 A. General Discussion

1. Uses of Toxicity Tests

Toxicity tests are desirable in water quality evaluations because chemical and physical tests alone are not sufficient to assess potential effects on aquatic biota.¹⁻³ For example, the effects of chemical interactions and the influence of complex matrices on toxicity cannot be determined from chemical tests alone. Different species of aquatic organisms are not equally susceptible to the same toxic substances nor are organisms equally susceptible throughout the life cycle. Even previous exposure to toxicants can alter susceptibility. In addition, organisms of the same species can respond differently to the same level of a toxicant from time to time, even when all other variables are held constant.

Toxicity tests are useful for a variety of purposes that include determining: (a) suitability of environmental conditions for aquatic life, (b) favorable and unfavorable environmental factors, such as DO, pH, temperature, salinity, or turbidity, (c) effect of environmental factors on waste toxicity, (d) toxicity of wastes to a test species, (e) relative sensitivity of aquatic organisms to an effluent or toxicant, (f) amount and type of waste treatment needed to meet water pollution control requirements, (g) effectiveness of waste treatment methods, (h) permissible effluent discharge rates, and (i) compliance with water quality standards, effluent requirements, and discharge permits. In such regulatory assessments, use toxicity test data in conjunction with receiving-water and site-specific discharge data on volumes, dilution rates, and exposure times and concentrations.

2. Test Procedures

There is a need to use correct terminology (see Section 8010B, Terminology), and environmentally relevant test procedures to meet regulatory, legal, and research objectives.³⁻⁸

The procedures given below allow measurement of biological responses to known and unknown concentrations of materials in both fresh and saline waters. These toxicity tests are applicable to routine monitoring requirements as well as research needs. Refer to Part 9000 for microbiological methods and Part 10000 for field and other types of biological laboratory methods for water quality evaluations. Refer to Section 10900 for identification aids for aquatic organisms.

Reasonable uniformity of procedures and of data presentation is essential. The use of standardized methods described below will ensure adequate uniformity, reproducibility, and general usefulness of results without interfering unduly with the adaptability of the tests to local circumstances.

Quality assurance practices for toxicity test methods include all aspects of the test that affect the quality of the data. These include sampling and handling, source and condition of test organisms, performance of reference toxicant tests, and the test procedures themselves. Quality assurance/quality control guidelines are available for single compound testing and general laboratory practices⁹ and for effluent evaluations in technical guidance manuals for conducting acute and short-term chronic toxicity tests with effluents.¹⁰⁻¹²

Other publications covering aspects of toxicity test procedures are available.^{8,13-15}

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* Approved by Standard Methods Committee, 1997.

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8010 B. Terminology

An aquatic toxicity test is a procedure in which the responses of aquatic organisms are used to detect or measure the presence or effect of one or more substances, wastes, or environmental factors, alone or in combination.

1. General Terms

Acclimate—to accustom test organisms to different environmental conditions, such as temperature, light, and water quality.

Response—the measured biological effect of the variable tested. In acute toxicity tests the response usually is death or immobilization. In plant toxicity tests, the response can be death, growth inhibition, or reproductive inhibition. In biostimulation tests, the response is biomass increase.

Control—treatment in a toxicity test that duplicates all the conditions of the exposure treatment but contains no test material.

Range-finding test—preliminary test designed to establish approximate toxicity of a solution. Test design incorporates multiple, widely spaced, concentrations with single replicates; exposure is usually 8 to 24 h.

Screening test—toxicity test to determine if an impact is likely to be observed; test design incorporates one concentration, multiple replicates, exposure 24 to 96 h.

Definitive test—toxicity test designed to establish concentration at which a particular end point occurs. Exposures for these tests are longer than for screening or range-finding tests, incorporating multiple concentrations at closer intervals and multiple replicates.

2. Toxicity Terms

Dose—amount of toxicant that enters the organism. Dose and concentration are not interchangeable.

Toxicity—potential or capacity of a test material to cause adverse effects on living organisms, generally a poison or mixture of poisons. Toxicity is a result of dose or exposure concen-

tration and exposure time, modified by variables such as temperature, chemical form, and availability.

Exposure time—time of exposure of test organism to test solution.

Acute toxicity—relatively short-term lethal or other effect, usually defined as occurring within 4 d for fish and macroinvertebrates and shorter times (2 d) for organisms with shorter life spans.

Chronic toxicity—toxicity involving a stimulus that lingers or continues for a relatively long period of time, often one-tenth of the life span or more. "Chronic" should be considered a relative term depending on the life span of an organism. A chronic toxic effect can be measured in terms of reduced growth, reduced reproduction, etc., in addition to lethality.

Lethal concentration (LCP)—toxicant concentration estimated to produce death in a specified proportion (P) of test organisms. Usually defined as median (50%) lethal concentration, LC50, i.e., concentration killing 50% of exposed organisms at a specific time of observation, for example, 96-h LC50.

Effective concentration (ECP)—toxicant concentration estimated to cause a specified effect in a designated proportion (P) of test organisms. The effect is usually sublethal, such as a change in respiration rate or loss of equilibrium. The exposure time also is specified; for example, the 96 h EC50 for loss of equilibrium is the effective concentration for 50% of the test organisms in 96 h, for this kind of effect.

Inhibition concentration (ICP)—toxicant concentration estimated to cause a specified percentage (P) inhibition or impairment in a qualitative biological function. For example, an IC25 could be the concentration estimated to cause a 25% reduction in growth of larval fish, relative to the control. Use this term with any toxicological test that measures a change in rate, such as respiration, number of progeny, decrease in number of algal cells, etc.

Asymptotic LC50—toxicant concentration at which LC50 approaches a constant for a prolonged exposure time.

Median tolerance limit (TLM)—test material concentration at which 50% of test organisms survive for a specified exposure time. This term has been superseded by median lethal concentration (LC50) and median effective concentration (EC50).

No-observed-effect concentration (NOEC)—in a full- or partial-life-cycle test, the highest toxicant concentration in which the values for the measured response are not statistically significantly different from those in the control.

Lowest-observed-effect concentration (LOEC)—in a full- or partial-life-cycle test, the lowest toxicant concentration in which the values for the measured response are statistically significantly different from those in the control.

3. Biostimulation Terms

Limiting nutrient—nutrient, among those required, which is inadequate in quantity for growth while others remain sufficient.

Nutrient—specific substance required for organism growth.

Maximum standing crop—maximum weight of organisms during a test, specified as wet or dry weight.

4. Solution Renewal Terms

Static test—test in which solutions and test organisms are placed in test chambers and kept there for the duration of the test.

Renewal test—tests in which organisms are exposed to solutions of the same composition that are renewed periodically during the test period (with renewals usually at 24-h intervals). This is accomplished by transferring test organisms or replacing test solution.

Flow-through test—test in which solution is replaced continuously in test chambers throughout the test duration.

5. Evaluation of Results

Maximum allowable toxicant concentration (MATC)—toxicant concentration that may be present in a receiving water

without causing significant harm to productivity or other uses. MATC is determined by long-term tests of either partial life cycle with sensitive life stages or a full life cycle of the test organism.

Chronic value (ChV)—geometric mean of the NOEC and LOEC from partial- and full-life-cycle tests and early-life-stage tests.

Acute-to-chronic ratio—numerical relationship between acute and chronic toxicity that is applied to acute toxicity test values to estimate toxicant concentration that is safe for chronic or long-term exposure of a test organism.

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8010 C. Basic Requirements

1. General Requirements

The basic requirements and desirable conditions for toxicity tests are: (a) an abundant supply of water of desired quality (see 8010E.4b), (b) an adequate and effective flowing water system constructed of nonpolluting or absorbing materials (see 8010F.1a), (c) adequate space and well-planned holding, culturing, and testing equipment and facilities (see 8010E.3), (d) an adequate source of healthy experimental organisms (see 8010E.4), and (e) appropriate lighting facilities for plant toxicity tests. Much valuable information and advice regarding general requirements and desirable conditions for toxicity testing are available.¹⁻⁹

2. Requirements for Specific Test Purposes

The facilities, equipment, and water supplies needed for effective tests depend on the type of tests and their objectives.⁶ For

effluent and monitoring compliance tests requiring receiving water as the dilution water, use water immediately upstream and outside the zone of influence of the waste. When studies require the use of laboratory-grade water, use a water supply free from pollution and one that provides for acceptable survival, growth, and reproduction of the aquatic test organisms to be studied. The most important requirements for designing a toxicity testing program are defining objectives of the study and establishing quality control practices, to ensure that the data are of sufficient quality to address the objectives and to ensure credibility.

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8010 D. Conducting Toxicity Tests

1. Types of Toxicity Tests: Their Uses, Advantages, and Disadvantages

Toxicity tests are classified according to (a) duration—short-term, intermediate, and/or long-term, (b) method of adding test solutions—static, renewal, or flow-through, and (c) purpose—effluent quality monitoring, single compound testing, relative toxicity, relative sensitivity, taste or odor, or growth rate, etc.

Short-term toxicity tests are used for routine monitoring suitable for effluent discharge permit requirements and for exploratory tests. They may use end points other than mortality. Acute definitive tests typically use mortality as an end point or other discrete observations to determine effects due to the toxicant (i.e., LC50 or EC50 values). These tests also may be used to indicate a suitable range of toxicant concentrations for intermediate and long-term tests. Short-term tests, rather than longer-duration tests, are used to obtain toxicity data as rapidly and inexpensively as possible. They are valuable for estimation of overall toxicity, for screening test solutions or materials for which toxicity data do not exist, for assessing relative toxicity of different toxicants or wastes to selected test organisms, or for relative sensitivity of different organisms to different conditions of such variables as temperature and pH. The results of these tests can be used to calculate acceptable concentrations for very short exposures, such as those that might occur as organisms pass through an effluent zone of initial dilution or a mixing zone.

Toxicity tests of intermediate duration typically are used when longer exposure durations are necessary to determine the effect of the toxicant on various life stages of long-life-cycle organisms, and to indicate toxicant concentrations for life-cycle tests.

Long-term toxicity tests are generally used for estimating chronic toxicity. Long-term testing may include early-life-stage, partial-life-cycle, or full-life-cycle testing. Exposures may be as short as 7 d to expose specific portions of an organism's life cycle, 21 to 28 d to several months or longer for traditional partial-life-cycle and full-life-cycle tests with fish.

To establish a successful testing program, consider the following: Use caution when static tests are used for evaluation of solutions with high BOD and/or COD levels or high bacterial

populations. These tests can be conducted successfully with incorporation of rigorous dissolved oxygen monitoring and acceptable aeration. Volatile or unstable toxicants may decrease in concentration during the test, resulting in an underestimation of the exposure concentration causing an effect on the test organisms. Metabolic products, such as ammonia, may increase to undesirably high concentrations resulting in stress or death of test organisms and overestimation of the concentration that causes a toxic response. Toxicant concentration may be reduced by sorption on sediments, test chamber walls, by the food provided for the test organisms, or by combination with the mucus or metabolic products of the test organisms and in their bodies.

Flow-through toxicity tests are desirable for high-BOD or COD samples and for those that contain unstable or volatile substances. Organisms with high metabolic rates are difficult to maintain under static exposure conditions, whereas flow-through tests provide well-oxygenated test solutions and continuous removal of metabolic wastes. Use flow-through toxicity tests whenever there is evidence or expectation of rapid degradation of the test solution. Such a change is indicated when the survival time of test animals in a fresh solution is significantly shorter than in a corresponding 2-d-old solution (provided that adequate DO is present throughout both tests). Flow-through toxicity tests are also desirable for industrial effluents and chemicals that are removed appreciably from solution by precipitation, by test organisms, or by other means.

The LC50 values may be useful measures of acute toxicity but they do not represent concentrations that are safe or harmless in aquatic habitats. Concentrations of wastes that are not demonstrably toxic in 96 h may be toxic at longer exposure periods in a receiving water. Thus the 96-h LC50 may represent only a fraction of long-term toxicity. When estimating safe discharge rates or dilution ratios for effluents or other pollutants on the basis of acute toxicity evaluations, use acute-to-chronic ratios determined primarily from life-cycle tests; however, NOEC values determined from shorter-duration chronic toxicity tests can be used. Even the provision of an apparently ample margin of safety can fail to accomplish its purpose when there is cumulative toxicity that cannot be predicted from acute toxicity results.

No single, simple acute-to-chronic ratio is valid for all wastes or toxicants. However, research on effluents has shown that acute-to-chronic ratios for whole effluents often are around 10. An acute-to-chronic ratio of 20 commonly has been used for nonpersistent chemicals while a factor of 100 has been used for persistent chemicals. The constituents of a complex waste responsible for acute toxicity may be, but are not necessarily, the constituents responsible for chronic or cumulative toxicity demonstrable in diluted waste that is no longer acutely toxic. The chronic toxicity may be lethal after a long exposure period or it may only cause impairment of function. Knowledge of acute toxicity of a waste often can be very helpful in predicting and preventing acute damage to aquatic life in receiving waters as well as in regulating toxic waste discharges.

2. Short-Term Toxicity Tests

a. Range-finding toxicity tests: For effluents or materials of unknown toxicity conduct short-term (usually 24-h or 48-h), small-scale range-finding or exploratory tests to determine approximate concentration range to be included in definitive short-term tests. For effluents with low or slow-acting toxicity, 48- or 96-h tests may be necessary. Expose test organisms to a wide range of concentrations of the test substance, usually in a logarithmic ratio, such as 0.01, 0.1, 1, 10, and 100% of the sample. Attempt to include concentrations that will kill all organisms and others that will kill very few or no organisms. For short-term, definitive tests, select a geometrically spaced series of concentrations between the highest concentration that killed no, or only a few, test organisms and the lowest concentration that killed most or all test organisms.

Prepare test concentrations as described in Section 8010F.2b.

b. Short-term definitive tests: Because death is an important, easily detected adverse effect, the most commonly used tests are for acute lethality. These tests are most appropriate for routine monitoring and checking conformity with NPDES requirements.¹ If it is not possible to perform a range-finding toxicity test before a definitive acute toxicity test, using a concentration series with a 0.5 (100, 50, 25, 12.5, 6.25%) or 0.3 (100, 30, 10, 3, 1%) dilution factor may be appropriate. Short-term tests may be static, renewal, or flow-through. Exposure periods for these tests usually are 48 h or 96 h. Static or renewal tests often are used when the test organisms are phyto- or zooplankton because these organisms are easily washed out in flow-through tests. Static and renewal tests are considerably less expensive to perform than flow-through tests. Overnight express mail shipments of samples often make static and renewal tests the method of choice for regulatory compliance testing.

Test solutions may be renewed daily if required because of oxygen demand, if the toxicant is unstable or volatile, or in the case of whole effluents, daily variation in the composition of the effluent. Renewals also may be less frequent. If the test material has high BOD and/or COD level or is relatively unstable, use test vessels with maximum surface area-to-volume ratio, or use the renewal or flow-through technique.

Test duration is determined by the toxicant and the test objectives and usually is the same for different groups of organisms. For short-life-cycle organisms such as phytoplankton, the usual exposure time can cover many generations. Determine test duration, in part, by the length of the life cycle. Generally,

expose fish and large invertebrates in static and static renewal tests for 96 h and in flow-through tests for an equal period unless composition of the toxicant is variable. In this case longer exposure may be useful to assess impacts of toxicant variability. Expose *Daphnia* and *Ceriodaphnia* for 48 h. Short-term tests have been limited arbitrarily to 96 h, but longer tests sometimes are desirable because death does not always occur within the 48-h or 96-h period. When some test animals, though still alive, are dying or evidently affected after 96-h exposure, prolong the test or express the results of the test as a 48-h or 96-h EC50, defining the observed effect. If tests are continued for longer periods, the test organisms may need to be fed.

Feed test organisms as directed in specific sections of Part 8000. Record feeding and ensure that it is equivalent in each container.

Special tests may be conducted on altered or treated samples of effluent to obtain additional toxicity information. For example, effluent dilution water mixtures may be aged 24 to 48 h before adding the test organisms, to determine changes in toxicity. When special tests are conducted, describe methods in detail.

3. Intermediate-Term Toxicity Tests

No sharp time separation exists between short- and intermediate- or between intermediate- and long-term tests. Usually tests lasting 10 d or less are considered short-term while intermediate tests may last from 11 to 90 d. The length of the test organism's life cycle helps to determine what is short-term, intermediate, or long-term for that species. Intermediate-length tests may be static, renewal, or flow-through, but flow-through tests are recommended for most situations. For conduct of tests see Section 8010F.3a.

4. Long-Term, Partial- or Complete-Life-Cycle Toxicity Tests

With few exceptions, use flow-through tests with exposure extending over as much of the life cycle as possible. Continue tests from egg to egg or beyond, or for several life cycles for smaller forms. Determine the maximum concentrations of toxicant not producing harmful effects with continuous exposure. The overall objective of this type of test is to determine NOECs or chronic value (ChV) of effluents, toxicants, or wastes. Use life-cycle tests whenever possible to determine acute-to-chronic ratios and the effects on growth, reproduction, development of sex products, maturation, spawning, success of spawning and hatching, survival of larvae or fry, growth and survival of different life stages, deformities, behavior, and bioaccumulation, although bioaccumulation (or bioconcentration) often is determined with more mature animals in specially designed tests.²

In life-cycle or partial-life-cycle tests, ensure that water quality factors such as temperature, pH, salinity, and DO follow the natural seasonal cycle unless the test objective is to study one of these factors. It may be essential that the natural annual cycle be duplicated if the development of sex products, spawning, and development of eggs and larvae are to be normal. Whenever possible, do not let toxicant concentrations vary by more than $\pm 15\%$ from the selected concentration because of uptake by test organisms, absorption, precipitation, or other factors.

In these tests, select five or more concentrations on the basis of short- or intermediate-term tests and set up the exposure chambers at least in duplicate. Vary exposure chambers, spawning chambers, and other equipment to meet the needs of the different organisms. (See Sections 8111 through 8910.) Other apparatus, water supplies, and analytical determinations are listed in Section 8010E.

5. Short-Term Tests for Estimating Chronic Toxicity

Tests are available to estimate long-term effects of a toxicant or effluent after a relatively short (7 d) exposure. End points for the tests, called chronic estimator or rapid bioassessment tests, include lethality, reproductive potential, and growth. Tests estimating chronic toxicity frequently are being included as bio-monitoring requirements in discharge permits. The long duration of life-cycle or early-life-stage chronic tests increases the cost and reduces ability of laboratories to conduct long-term tests successfully as the demand for testing increases. The EPA has published a number of short-term chronic estimation toxicity test methods for fresh- and saltwater invertebrates and fishes.^{3,4} These tests were designed to evaluate effluent toxicity and may not be appropriate for other testing requirements such as pre-manufacturing testing, development of water quality criteria, etc.

6. Special-Purpose Toxicity Tests

a. Relative sensitivity to a toxicant: To rank the sensitivity of different species to a toxicant, use a standard water and standard exposure conditions. Select exposure conditions (e.g., temperature, DO, pH, CO₂, light, and salinity) in a favorable range for the test species and keep conditions constant throughout the test.

b. Relative sensitivity of various toxicants to selected species: These tests resemble sensitivity tests because the selected test conditions, dilution waters, and test species are kept constant and standard. Prevent any change in sensitivity of test organisms during the tests. If possible, select species from several different groups: an alga, microcrustacean, macrocrustacean, insect, mollusk, or fish.

c. Toxicity reduction evaluation: Use acute and chronic toxicity tests to determine the toxicant in the effluent. A Toxicity Reduction Evaluation (TRE) is a phased approach that first, characterizes the acute or chronic toxicity of an effluent, second, identifies the toxicant(s) of concern (this phase often is termed Toxicity Identification Evaluation, TIE), and third, confirms toxicity. This approach is then used to evaluate the removal of the toxicant(s) by pretreatment or changes at the wastewater treatment plant. EPA guidance manuals for performance of TIEs⁵⁻⁹ and generalized TRE protocols for municipal¹⁰ and industrial¹¹ facilities are available. Other TRE protocols are available.¹²⁻¹⁶

d. Flesh tainting tests: CAUTION: Perform such tests only when there is assurance that the intake of potentially tainting substances through consumption of the organism is safe. In cases where sufficient information on the substances is not available, replace consumption with smell. Use these tests to determine the maximum concentrations of wastes and materials that do not taint the flesh of edible aquatic organisms. Expose organisms that are large enough to supply portions for a taste panel. Set up exposure tanks as for other flow-through tests. Perform range-

finding tests over a wide concentration range to determine the concentrations for a more definitive series of tests.

After exposure, prepare test organisms for taste testing. Clean, prepare for cooking (without seasoning), wrap in aluminum foil, and bake in an oven. When organisms are cooked, divide them into portions, wrap in aluminum foil, assign a code number, and distribute to a taste panel while still warm, along with samples of unexposed organisms similarly cooked, wrapped, and coded. Record the observations of the panel on a prepared form and determine the highest concentration of test material not causing detectable tainting based on either taste or smell. Several tests may be necessary.

e. Growth-rate determinations: Growth rate is an important response of both algae and fish to toxicants and environmental factors. This section discusses the topic with respect to fish. For a discussion related to algae see Section 8111G.3c. Always report details of the method of feeding fish in growth studies. Three techniques are available:

Unrestricted food supply—Provide attractive and palatable food (usually live food such as *Daphnia*, tubificid worms, or brine shrimp) continuously in greater quantities than fish can consume. It is desirable to make a mass balance on food consumed by weighing food introduced and uneaten food removed.

Intermittent satiated food supply—Provide all the attractive food that fish can consume at time of feeding once or twice daily. After fish cease to feed, remove all uneaten food.

Uniformly restricted food supply—Once or twice per day, provide all fish with an amount of food that they will consume completely and without exception. Ideally, hold fish separately in individual aquariums or compartments. For fish held together, feed so that all fish have an equal opportunity to consume food. Uniformity of temperature and DO helps to ensure equal feeding of a group of fish.

While growth studies usually have been conducted with unrestricted and intermittent satiated feeding techniques, it is recommended that each study include at least one test series using uniformly restricted food supply. Only this technique can reveal whether growth rate differences are not the result of the effect of the toxicant on appetite or food consumption rate. The presence of an abundant food supply can obscure toxic effects. For example, fish exposed to toxicants such as cyanide or pentachlorophenol increase food consumption rate to compensate partially for loss of efficiency of food utilization caused by the toxicant. This may not be possible in natural conditions where food supply may be limited.

Ideally, include a series of tests with different, uniformly restricted food rations with the lowest ration near that which results in no growth (or loss of weight) in the control. This is the maintenance level. Determine the effect of the variable under study at any level of food availability and consumption by relating observed growth rates to, for example, toxicant concentration, at each feeding level.

Juvenile fish may gain enough weight in 1 to 3 weeks to determine growth rate satisfactorily. Longer exposures with weighings at intervals of approximately 10 d are needed to determine long-term effects such as acclimation or accumulative toxicity.

Report results as specific growth rates computed as follows:

$$\text{Growth rate} = \frac{\text{Weight gain, g}}{\text{time interval, d}} \times \frac{1}{\text{mean weight, g}}$$

where:

$$\text{mean weight} = [\text{weight at start of time interval (g)} \div \text{weight at end of time interval (g)}] \div 2$$

Determine dry weight, wet weight, and fat (lipid) content of fish at the beginning and end of a test. Weight gain due to increased fat content is not universally considered true growth; some investigators consider that true growth occurs only when there is an increase of protein. However, fat storage is important ecologically and bioenergetically because fat can be used as an energy source during periods of malnutrition, reproduction, and overwintering survival. Fat content also is important in the dynamics of toxicant uptake, storage, and depuration. Fat and water content should typify that of the target species.

7. References

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8010 E. Preparing Organisms for Toxicity Tests

1. Selecting Test Organisms

The prime considerations in selecting test organisms are: their sensitivity to the factors under consideration; their geographical distribution, abundance, and availability within a practical size range throughout the year; their recreational, economic, and ecological importance and relevance to the purpose of the study;

their abiotic requirements and whether these requirements approach the conditions normally found at the study site; the availability of culture methods for rearing them in the laboratory and a knowledge of their physiological and nutritional requirements; and their general physical condition and freedom from parasites and disease. To select a best species consider available information on sensitivity, consult with local authorities in pol-

lution control or fish and wildlife agencies, or determine sensitivity with short-term tests. Select the test species based on the considerations listed above as well as organism size and life-cycle length. Sections 8110 through 8910 list plant, invertebrate, and fish species that are commonly used in aquatic toxicity testing. For testing of early life stages of organisms, species having a short life cycle are most cost-effective, but some tests require larger organisms with long life cycles (e.g., bioaccumulation or in situ biomonitoring studies).

For studies to determine effluent effects, select species representative in the area impacted. In most cases, use of laboratory-cultured species is preferable to use of those collected from the field. Laboratory-cultured organisms, either from in-house cultures or purchased from commercial bioassay organism suppliers, are of known age and quality (those taken from the field may already have been selected or biased for the more resistant members of the population). This allows for use of the most sensitive life stages throughout the year. Their use also may be more cost-effective and allows for better quality assurance and control. For each series of tests, use organisms from a single source. Choose organisms that are nearly uniform in size, and for fish, with the largest individual not more than 50% longer than the shortest. Use organisms of the same age group or life stage. Optimally, conduct reference toxicant tests on cultured stocks and on lots of acquired or collected organisms.¹ Report time, place, source, and culture history for cultured organisms and method of collection, transportation, handling, and acclimation of acquired or collected organisms, and their response to reference toxicants.

Knowledge of their environmental requirements and food habits is important in selecting test organisms. Methods for laboratory holding and culturing are well described for a number of standard test species. When the purpose of the testing is site-specific, it may be necessary to collect certain life stages of selected organisms from the field for testing.

2. Collecting Test Organisms

Preferably, use standard laboratory-reared organisms. In special instances, such as when it is important to incorporate genetic variability from wild populations, use species indigenous to the receiving water. This is particularly important for organisms with recreational, commercial, or ecological significance.

In designing a test, consider any unusual past conditions to which the organisms may have been exposed (pesticides, effluents from industries, waste treatment plants, return flows, etc.). Interactive effects of a new toxicant mixed with those presently being discharged to the receiving water may be important. Do not collect test organisms from polluted areas where they are in poor condition, diseased, parasitized, or deformed, or where they have unusually high body burdens of chemicals. Avoid testing organisms with questionable histories.

Many smaller invertebrates and fish can be collected along the shore in dip nets, in coarse plankton nets, or by hand. Catch larger species that occur near shore in seines. Traps, fyke nets, and trawls are valuable tools for collecting organisms, but may be selective for some species. Otter trawls are effective for collecting benthic species and midwater trawls for pelagic species. Various dredges are available to collect benthic species from different types of bottoms or to collect different sizes of

organisms. Commercially important species such as lobster, blue crab, and dungeness crab may be taken by the researcher in traps or deep-water trawls or may be purchased from commercial suppliers if proper care is taken before purchase. Species that colonize surfaces, such as barnacles, may be harvested from hard surfaces submerged in the water. Insure that organisms are not damaged during collection, transfer, and transport.

When seining or using trawls, make short hauls. Avoid collecting significant amounts of plant materials, debris, mud, sand, or gravel in net or in bag of seine because these will injure the animals. Always leave seine bag in the water at end of haul, stretch out wings of seine, open bag entrance, dip out organisms with a bucket or hand net, and transfer directly to prepared holding tanks. Do not expose delicate, easily damaged species to air. Take out larger, more hardy species with soft mesh dip nets. Do not collect too many animals at one time. After bringing a trawl up to the boat, bring it over the side without delay and avoid letting the catch hit the boat. Immerse that portion of net containing specimens in a tank of water. Open trawl and remove desired animals by dipping with a bucket or a hand net with small soft mesh. Have adequate quantities of clean water available in tanks before beginning a haul. Transfer organisms to tanks as rapidly and carefully as possible.

If organisms are to be transported any distance by boat, hold in aerated live boxes. If they are transported by truck, put them in large baffled and insulated tanks filled with water from area in which they were collected. Aerate the water and maintain at temperature of collection. Determine water temperature, salinity, DO, and pH at the collecting site. Do not handle organisms more than necessary. Make transfers with suitable containers or hand nets, or for small organisms, by large-bore pipets. Use hand nets made of soft material with several layers around the net rim and free from sharp points or projections. Clean and sterilize all equipment before use. Avoid overcrowding of organisms during transport. Aeration, oxygenation, water exchange, and cooling may reduce distress. Avoid cold shock as much as overheating.

Observe collected animals for possible injury resulting from transport to the laboratory. Examine smaller forms under a dissecting microscope. Criteria for assessing injury depend on the species and are more difficult for sluggish ones. Useful criteria include lost or damaged appendages, inability to maintain a normal body posture (e.g., dorsal side uppermost), abnormal locomotion, refusal to feed, discoloration, or uncoordinated movements of the mouth or other body parts.

For additional information on collecting aquatic organisms, see Part 10000.

3. Handling, Holding, and Conditioning Test Organisms

During transport to the laboratory, organisms sometimes are crowded, bruised, and otherwise stressed, thereby increasing their susceptibility to disease. To avoid outbreaks of disease in stock tanks, treat organisms during transit or on arrival in accordance with procedures in ¶ 5 below and as suggested for each of the different groups (Sections 8211 through 8910). Hold field-collected fish in quarantine for at least 7 d to observe for parasites and disease, and to recover from collection and transport stress; observe invertebrates for at least 2 d. If more than 10% of the collected animals die after the second day or if they are parasitized or diseased beyond control, do not use them.

Clean and sterilize all contacted containers and equipment and collect another supply from a different area if possible.

Because it is not always possible to collect from unpolluted areas and the collector cannot always be sure that a particular organism has not been exposed to a toxicant, for certain types of tests it may be necessary to sample collected individuals to determine if they have accumulated pesticides, heavy metals, and toxic materials to be studied. Check animals or materials collected as food for test organisms for disease and content of pesticides, heavy metals, and toxic materials to be studied. Feed test organisms daily during quarantine.

After quarantine period, transfer disease-free animals to regular stock tanks. Discard organisms that touch dry surfaces, are dropped, or are injured during handling. To avoid unnecessary stress, do not subject organisms to rapid temperature or water-quality changes. In general, change water temperature less than 3°C in any 24-h period. For stenothermal, deep-water species use an even smaller rate of temperature change. Preferably keep DO concentrations at or near saturation but never at less than 60% or greater than 100% of saturation. After transfer to stock holding tanks, begin a slow acclimation to laboratory conditions such as temperature, salinity, and hardness. The period of acclimation will be governed by type of organism and extent of changes in water quality. For forms with a life cycle of several months or more, use an acclimation period of at least 2 to 3 weeks.

Inspect organisms closely and frequently to determine stress, unusual behavior, parasites or disease, changes in color, or failure to eat. Avoid crowding. Provide adequate flow-through water so that characteristics such as DO, pH, CO₂, salinity, hardness, and NH₃ are favorable. Check temperature and DO frequently. Do not let metabolic products accumulate. Generally, use a flow-through rate of 6 to 10 tank volumes/d. Usually, greater amounts of flow-through water are required for smaller organisms on a weight-volume basis. For small organisms, use a water flow of at least 3 L/d/g. When brood stock are being held, periodic or continuous treatment for parasite and disease control may be required.²⁻¹⁰

Clean tanks and equipment thoroughly and often, removing or flushing out all growths and wastes, preferably daily but at least twice per week. Remove all uneaten food within 24 h. Use different sets of nets and other equipment for different groups of organisms and clean and sterilize them between uses. When handling is necessary, clean hands and nets before touching organisms. Cover tanks and containers to prevent organisms from jumping out. Shield tanks with curtains or by some other means to protect organisms from unnecessary disturbances and noise. Provide photoperiods and light intensities favorable to the organisms (see Section 8010F.3f). Begin acclimation to test conditions at a suitable interval in advance of testing.

It is of utmost importance that animals be kept in excellent condition before the tests. Make no abrupt changes in environmental conditions; preferably follow natural seasonal variations in environmental conditions such as temperature and daylight patterns. Many water supplies are supersaturated with gases, especially in winter when very cold water is brought into the laboratory and warmed. Because there is a danger of gas bubble disease, keep incoming water in an open system and let it cascade over baffles or otherwise aerate it to bring dissolved gases into equilibrium with the air or strip supersaturated nitrogen with pure oxygen.¹¹

Acclimate freshwater arthropods by rearing them in the dilution water at the test temperatures, unless temperature is one of the factors being studied. Acclimate other organisms to the dilution water and test temperatures by gradually changing the water from 100% holding water to 100% dilution water over a period of several days. Keep all organisms in 100% dilution water for at least 2 d before use. Do not use a group of organisms if more than 10% die during the 48 h immediately before the beginning of the test.¹ If a group fails to meet these criteria discard or re-treat, hold, and reacclimate if necessary.

Make necessary provisions for organisms that require a special substrate, cover, or materials to use for clinging, support, the building of cases, or hiding.

Hold cold-water, freshwater organisms between 5 and 15°C. Hold warm-water organisms between 10 and 25°C, depending on season and test objectives.

4. Culturing Test Organisms

The advantage of cultured test organisms over field-collected animals is that the age, life history, and existing conditions are documented and thus the responses of these organisms are more consistent between test lots. For organisms used extensively in effluent biomonitoring programs, EPA has developed a series of test methods^{12,13} that are adaptable to most laboratories. Culturing test organisms requires strict adherence to standard protocol, 7-d/week monitoring, and an adequate facility.

a. Facilities, construction materials, and equipment: Do not use construction materials in contact with dilution water that contain leachable substances or adsorb significant amounts of substances from the water. Use tempered glass, fiberglass, or stainless steel (No. 316) and silicone sealant as construction material for freshwater systems. Do not use rubber or plastics containing toxic fillers, additives, stabilizers, plasticizers, etc. Fluorocarbon plastic, nylon, and their equivalents usually are acceptable. Test the toxicity of all materials before purchasing large quantities. Clean, soak, and flush all new tanks, troughs, and similar equipment with dilution water for several days before use. Use a glass or titanium interface between the water and heating elements for marine waters and glass or stainless steel for fresh waters.

Provide adequate space for test organisms, holding facilities, water storage reservoirs, and water supply systems. Provide distribution of hot and cold water and mixing facilities to obtain any desired temperature. Aerate or vigorously mix to prevent gas supersaturation caused by heating dilution water.⁴ Use oil-free pumps if possible. If air pumps are not oil-free, they should have water seals and filters to prevent oil from entering air lines and contaminating tanks. When large volumes of air are needed, use low-pressure blowers. Do not locate air intakes in shops or furnace rooms or near outlets from chemical exhaust hoods, chemical laboratories, or vehicle exhausts.

Provide acclimation and culturing tanks with temperature control and aeration. Design holding facilities for ease of cleaning and prevention of bacterial growths. For holding and culturing fish and many macroinvertebrates, preferably use round or oval tanks of at least 1 to 3 m diam (Figure 8010:1). Provide a standpipe drain in the center, threaded below the tank floor so that, when the standpipe is removed, the opening is flush with the tank bottom. Slope tank bottom gently to center. Use tanks with

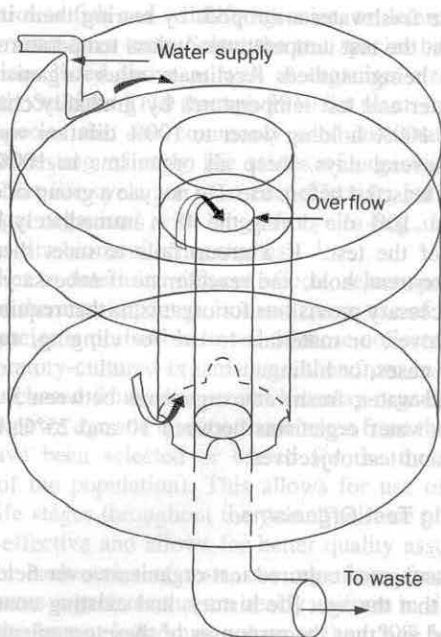


Figure 8010:1. Holding tank design for fish and macroinvertebrates.

smooth surfaces to facilitate cleaning, to prevent injuries to organisms, and to insure that no material will collect in corners, cracks, and crevices. Introduce water into a circular tank as a jet along the edge and above the surface to create a circular movement of water around the central standpipe. Fit another pipe, with half-moon cutouts at its base, over the standpipe and screen, so that the outflowing water passes up through the outside pipe, then down the standpipe. This results in a circular current and a certain amount of self-cleaning. Square or rectangular tanks may be used for special purposes or when space is scarce. Provide standpipes at one end for draining, with threads for securing the pipe on the underside. Ensure that tank corners are rounded and that surfaces are smooth.

b. Water supply: Provide a flowing water system for holding, spawning, and rearing a variety of aquatic organisms. In general, reconstituted fresh water and artificial seawater are not cost-effective for large-scale rearing or for flow-through tests. If available, use natural, unpolluted fresh or salt water supplies that have low turbidity, high DO, low BOD, and an annual temper-

ature cycle that approximates that of the test organisms. If a flow-through water supply is not available, use of reconstituted fresh water or artificial salt water in recirculating culture systems with biological filters may be acceptable.

1) **Freshwater supplies**—A good freshwater supply is constant in quality, provides for adequate survival, growth, and reproduction of test organisms, and does not contain more than the designated amounts of the following: suspended solids, 20 mg/L; total organic carbon (TOC), 2 mg/L; chemical oxygen demand (COD), 5 mg/L; un-ionized NH_3 , 20 $\mu\text{g/L}$; total residual chlorine, 0.01 mg/L; total organophosphorus pesticides, 50 ng/L;* total organochlorine pesticides plus PCBs, 50 ng/L. Consider water to be of constant quality if the monthly ranges of hardness, alkalinity, conductivity, TOC or COD, and salinity are less than 10% of the average values and the pH range is less than 0.4 units.

Check municipal water supplies to determine their acceptability from the standpoint of, for example, copper, lead, zinc, fluoride, and free and combined chlorine concentrations; these are typical concerns, but others may be equally relevant. If a satisfactory freshwater supply is not available or if a standard water is required for comparative toxicity tests, relative sensitivity tests, or tests to determine the effects of hardness, pH, or total alkalinity on the toxicity of various materials, use a reconstituted standard water. Gas supersaturation is common in nearly all water supplies and merits concern. Total dissolved gas levels should not exceed barometric pressure in shallow tanks (≤ 1 m deep) and should be monitored as described in Section 2810. Supersaturated gas levels can be reduced by aeration⁴, but complete removal requires pretreatment by nitrogen stripping with pure oxygen.

Prepare standard fresh water (Tables 8010:I and 8010:II) by adding reagent-grade chemicals to glass-distilled and/or deionized water. For special studies, determine that the reverse osmosis, distilled and/or deionized water contains less than the indicated constituents:

Conductivity	1 $\mu\text{S/cm}$
Total organic carbon (TOC)	2 mg/L
or chemical oxygen demand (COD)	5 mg/L
Boron, fluoride	100 $\mu\text{g/L}$ each
Un-ionized ammonia	20 $\mu\text{g/L}$

* No individual pesticide should exceed the allowable concentration limit set in the National Water Quality Guidelines, EPA, as set in accordance with the Federal Water Pollution Control Act 92-500 as amended 1972.

TABLE 8010:I. RECOMMENDED COMPOSITION FOR RECONSTITUTED FRESH WATER

Water Type	Salts Required mg/L				Water Quality		
	NaHCO_3	$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	MgSO_4	KCl	pH*	Hardness mg CaCO_3/L	Alkalinity mg CaCO_3/L
Very soft	12	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48	30	30	2.0	7.2-7.6	40-48	30-35
Moderately hard	96	60	60	4.0	7.4-7.8	80-100	60-70
Hard	192	120	120	8.0	7.6-8.0	160-180	110-120
Very hard	384	240	240	16.0	8.0-8.4	280-320	225-245

* Approximate equilibrium pH after aeration with fish in water.

TABLE 8010:II. QUANTITIES OF REAGENT-GRADE CHEMICALS TO BE ADDED TO AERATED SOFT RECONSTITUTED FRESH WATER FOR BUFFERING pH^{5,8}

Desired pH*	Quantity of Chemical to Be Added mL/L water		
	1.0N NaOH	1.0M KH ₂ PO ₄	0.5M H ₃ BO ₃
6.0	1.3	80.0	—
6.5	5.0	30.0	—
7.0	19.0	30.0	—
7.5	—	—	—
8.0	19.0	20.0	—
8.5	6.5	—	40.0
9.0	8.8	—	30.0
9.5	11.0	—	20.0
10.0	16.0	—	18.0

* Approximate equilibrium pH. Do not aerate after adding these chemicals.

Aluminum, arsenic, chromium, cobalt, copper, iron, lead, nickel, zinc 1 µg/L each
Total residual chlorine < 10 µg/L
Total organophosphorus pesticides 50 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs) 50 ng/L

Carbon-filtered deionized water usually is acceptable. Determine conductivity of distilled and/or deionized water for each batch of reconstituted water. Check other constituents periodically. If the water is prepared from a dechlorinated water, test the reconstituted water to determine that first instar daphnids survive for 48 h and can reproduce successfully when mature (Section 8711).¹

The pH, alkalinity, and hardness of a receiving water influence toxicity of some materials, especially metals. Therefore, it is desirable to have a supply of both hard and soft waters with suitable pH and alkalinity.

It is advantageous to have water with temperatures between 3 and 12°C during the winter and between 12 and 25°C at peak summer temperatures. For general use, the pH should be in the range of 7 to 8.2 and dissolved CO₂ should be 1 mg/L or less.

2) Marine water supplies—Use unpolluted marine water with low turbidity and settleable solids and a pH and salinity favorable for the test organism. Ensure that annual salinity variations are not so wide as to be harmful to the organisms. In general, it is preferable to have a source of higher-salinity water (e.g., ocean water) from which brackish water can be prepared by dilution.

If a suitable marine water supply is not available, use artificial seawater for limited culturing and toxicity testing.¹⁵⁻¹⁷ Prepare artificial seawater by adding the compounds listed in Table 8010:III to 800 mL glass-distilled or deionized water, in the order listed. Make sure that each salt is dissolved before adding the next. Make up to 1 L with distilled or deionized water. The salinity should be 34 ± 0.5 g/kg and pH 8.0 ± 0.2. Obtain desired salinity at time of use by dilution with deionized water. Alternatively, prepare marine water from commercially available salt mixes that have been shown to provide for adequate survival, growth, and reproduction of test organisms.

To increase salinity of a natural water, use a strong natural brine prepared by freezing and then partially thawing seawater.

TABLE 8010:III. PROCEDURE FOR PREPARING RECONSTITUTED SEAWATER*^{15,17}

Compound in Order of Addition	Final Concentration mg/L
NaF	3
SrCl ₂ ·6H ₂ O	20
H ₃ BO ₃	30
KBr	100
KCl	700
CaCl ₂ ·2H ₂ O	1 470
Na ₂ SO ₄	4 000
MgCl ₂ ·6H ₂ O	10 780
NaCl	23 500
Na ₂ SiO ₃ ·9H ₂ O	20
Na ₄ EDTA*	1
NaHCO ₃	200

* Tetrasodium ethylenediaminetetraacetate. Omit when toxicity tests are conducted with metals. Omit when tests are conducted with plankton or larvae. Strip the medium of trace metals.^{11,12}

Natural brine also may be prepared (not to exceed 100‰ by evaporation of natural seawater with aeration and low heat (maximum of 35°C).¹³ This is satisfactory if only limited amounts of water are needed; for larger volumes, use commercial sea salts or a stronger solution of artificial seawater.

In the preparation of artificial seawater, be sure that an undesirable concentration of metals does not occur. Even reagent chemicals contain traces of several metals and their extensive use can result in a buildup of metals. If large volumes of artificial seawater are not required, remove the metals by passing the seawater through a column containing a cation-exchange resin in the sodium form.

The suitability of any artificial saltwater is enhanced by aging, with aeration, and by the introduction of nitrogen-fixing bacteria from water in which healthy aquatic organisms have been maintained or by addition of marine algae.

c. Food and feeding:

1) Culture of microorganisms—Phytoplankton and zooplankton may be cultured as test organisms for studying biostimulation, toxicity, etc. They may be cultured also as food for other organisms such as copepoda, *Daphnia* and other microcrustaceans, the larvae and adults of mollusks, and young and adult fish.

a) Culture medium for freshwater algae—Prepare reconstituted fresh water by adding reagent-grade macro- and micronutrients to glass-distilled and/or deionized water in the concentrations given in Tables 8010:IV.A and B.

Prepare a separate stock solution of each macronutrient salt at 1000 times the specified final concentration in glass-distilled or deionized water. Combine trace metals and EDTA in a single micronutrient stock solution in glass-distilled or deionized water at 1000 times the final concentration of each. Note that in toxicity testing of metals the effect of EDTA must be established and reported as part of the test results.

To prepare algal culture medium, add 1 mL of each macronutrient stock solution to 900 mL glass-distilled or deionized water, then add 1 mL micronutrient stock and make up to 1 L with glass-distilled or deionized water. As an alternative to algal

TABLE 8010:IV.A. MACRONUTRIENT STOCK SOLUTION

Compound	Concentration mg/L	Element	Resulting Concentration mg/L
NaNO ₃	25.5	N	4.20
		Na	11.0
NaHCO ₃	15.0	C	2.14
K ₂ HPO ₄	1.04	K	0.469
		P	0.186
MgSO ₄ ·7H ₂ O	14.7	S	1.91
		Mg	2.90
MgCl ₂	5.70		
CaCl ₂ ·2H ₂ O	4.41	Ca	1.20

TABLE 8010:IV.B. MICRONUTRIENT STOCK SOLUTION

Compound	Concentration μg/L	Element	Resulting Concentration μg/L
H ₃ BO ₃	186	B	32.5
MnCl ₂	264	Mn	115
ZnCl ₂	3.27	Zn	1.57
CoCl ₂	0.780	Co	0.354
CuCl ₂	0.009	Cu	0.004
Na ₂ MoO ₄ ·2H ₂ O	7.26	Mo	2.88
FeCl ₃	96.0	Fe	33.0
Na ₂ EDTA·2H ₂ O	300	—	—

culture medium preparation, commercially supplied nutrient media† are available.

To prepare duckweed culture medium, add 10 mL of each macronutrient stock solution to 900 mL glass-distilled or deionized water, then add 1 mL trace metal EDTA mixture and make up to 1 L. Alternatively, mix 10 mL each of three stock solutions (A, B, and C) to 1 L (8211B.2).

Whenever axenic algal cultures are used, or if bacterial growth interferes with the test, prepare media aseptically: To 800 mL glass-distilled or deionized water add 1 mL of each macronutrient stock solution in the order listed, mixing after each addition. Filter-sterilize by passing through a sterile 0.2-μm-porosity membrane filter (pre-rinsed with 100 mL double-distilled water) into an autoclave-sterilized container. Add 1 mL filter-sterilized micronutrient solution, and make up to 1 L with sterile distilled or deionized water.

Store uninoculated sterile reference medium in the dark to avoid photochemical changes.

When sterility is desired in algal tests, check sterility periodically by adding 1 mL inoculated test culture to tubes of sterile nutrient test medium and incubate in the dark at the test temperature for 2 weeks. The appearance of opalescence in the test medium indicates contamination.

Prepare sterile bacterial nutrient test medium by adding the following quantities of chemicals to 1 L glass-distilled water:

Sodium glutamate	250 mg
Sodium acetate	250 mg

Glycine	250 mg
Sucrose	250 mg
Sodium lactate	250 mg
D,L alanine	250 mg
Nutrient agar	50 mg

Bring medium to a boil, dispense to test tubes, and sterilize by autoclaving.

b) Culture medium for marine algae—To artificial seawater (Table 8010:III) add nutrients listed in Table 8010:V to give the indicated concentrations in the algal culture medium.

When an unpolluted seawater is used, prepare the medium by enriching filter-sterilized seawater with micronutrients at one-half the indicated concentrations. If sterile techniques are required, follow procedures for fresh water. When sterilization is performed by autoclaving, add vitamins after autoclaving. When filter-sterilization is used, use a positive pressure of 72 kPa.

2) Mass production of algae as food for other organisms—Rearing zooplankton, various filter-feeders, the larvae of crustaceans, and fish requires large quantities of phytoplankters. These needs must be met with an apparatus capable of producing continuous amounts of desired organisms at high densities. Such an apparatus (Figure 8010:2) permits easy assembly, cleaning, and sterilization and efficient utilization of light energy, and is constructed for continuous use. The main body of the unit is a 60-cm section of 15-cm-diam borosilicate glass drainage pipe. The top section is a 15-cm to 5-cm concentric reducer and the bottom section is a 15-cm to 5-cm ell. Each section accommodates a No. 12 silicone rubber stopper held in place by a carboy or similar type clamp. Hold the three sections together by aluminum ring clamps and seal adjoining surfaces by silicone "O" rings made from small-diameter tubing. Use material that is autoclavable and nontoxic.

Use this or similar device to supply cells on a periodic or continuous basis. As the cells are withdrawn, add more medium. The following species have been grown at the indicated concentrations: *Skeletonema costatum*, 4.3×10^6 cells/mL; *Dunaliella tertiolecta*, 4.4×10^6 cells/mL; *Isochrysis galbana*, 7.0×10^6 cells/mL; *Monochrysis lutheri*, 5.0×10^6 cells/mL.

3) Food for macroinvertebrates and fish—A suitable food is essential for rearing various macroinvertebrate and early life stages of some fishes. Distinguish carnivores from herbivores to

TABLE 8010:V. NUTRIENTS FOR ALGAL CULTURE MEDIUM IN SEAWATER

Compound	Concentration	Concentration of Nutrient
NaNO ₃	25.0 mg/L	4.2 mg N/L
K ₂ HPO ₄	1.05 mg/L	0.19 mg P/L
FeCl ₃	72.6 μg/L	
MnCl ₂	2.30 μg/L	
ZnCl ₂	2.10 μg/L	
Na ₂ MoO ₄ ·2H ₂ O	2.50 μg/L	
CuCl ₂	0.20 μg/L	
Na ₂ EDTA	300 μg/L	
Vitamins:		
Thiamine	0.100 mg/L	
Biotin	0.50 μg/L	
B ₁₂	0.50 μg/L	

† Fritz Chemical Co., Dallas, TX, or equivalent.

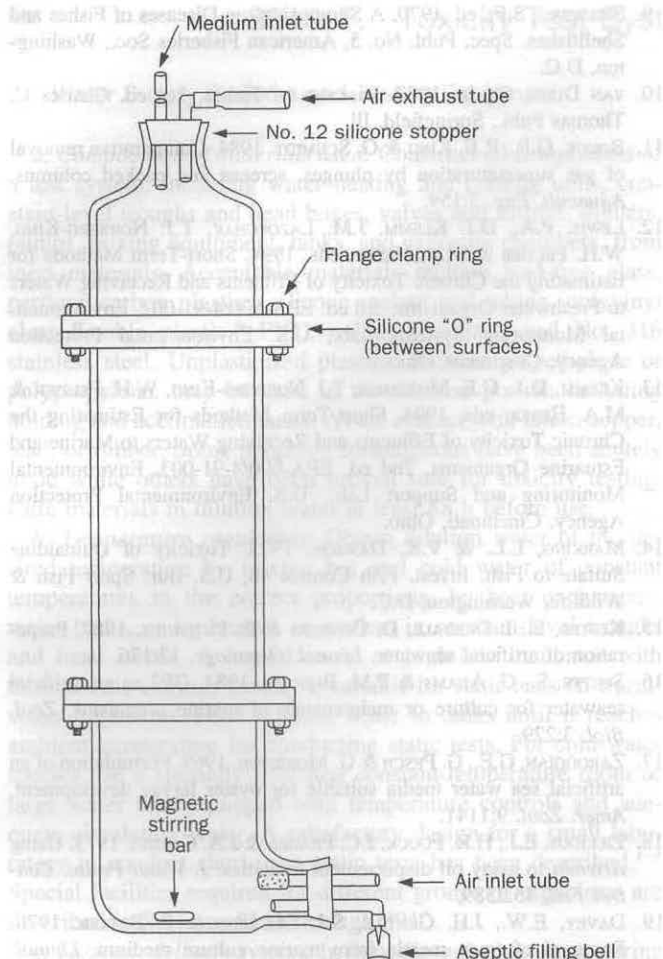


Figure 8010:2. Algal culture units. Cells are withdrawn from unit through the aseptic filling bell.

supply the correct type of food. Organisms taken as food differ for different life stages of a species. As organisms grow they require progressively larger food organisms. Many feed on pelagic organisms whose movements should be sufficient to attract the predator but slow enough so they can be caught readily. Use food organisms that are nutritious, easily digested, uncontaminated, and readily obtainable. The nutritional requirements of salt- and freshwater organisms can differ greatly; check specific requirements of particular species. Distribute zooplankton food in rearing tanks to match distribution of organisms using it. Provide an adequate amount of food with a ratio of number of prey to predator varying from 50:1 to 200:1, depending on the feeding efficiency of the cultured species. If a small number of organisms is reared in a large tank, provide an acceptable food organism density to insure that enough are captured. Some algae and diatoms used for food have a tendency to settle to the bottom. Circular movement of the water in rearing tanks, provided as described in Section 8010E.4a, keeps food materials in suspension.

When using cultured microorganisms as food, be aware of possible environmental changes they may cause. In addition to the possible presence of toxic metabolites, algal blooms may produce excess oxygen and result in supersaturation and gas

bubble disease.¹¹ Use live food whenever possible. Analyze food for toxicants, especially pesticides and heavy metals. Supplement natural foods with commercially available dried and pelleted foods.† (See Section 8910 for fish feeding.) These foods should be attractive to the organisms, supply necessary nutrients and trace elements, and contain binders to insure pellet stability.²⁰ Nutritionally deficient diets may cause significant differences in sensitivity of test organisms to toxicants and will affect reproductive performance.

Methods for rearing freshwater organisms have been described.²¹ Methods for rearing larvae of marine animals with special reference to their food organisms have been summarized.²² The literature on laboratory feeding larvae of marine fish²³ and freshwater fish²⁴ has been reviewed. Standard fish hatchery culture facilities and operations and other useful freshwater and marine aquaculture references are available.²⁵⁻²⁷

d. Cleaning containers and equipment:

1) Cleaning holding, acclimation, testing, and dilution water tanks—Clean test containers and toxicant delivery systems before use. Soak new solvent and acid resistant containers overnight in tap or dilution water, then wash with laboratory detergent, rinse with 100% acetone, water, acid (such as 5% HNO₃ or HCl), and rinse twice with tap water. After each test, wash the system appropriately, e.g., acid to remove metals and bases; detergent, sodium hypochlorite§ (NaOCl) solution (200 mg/L), organic solvent, or activated carbon to remove organic compounds, etc. Immediately before testing, rinse again with dilution water.¹

2) Removal of unused food and wastes—Do not let unused food or fecal material accumulate. Whenever possible, build holding and testing containers with sloping bottoms so food and feces can be removed easily with a siphon. The amount and frequency of cleaning depends on the organism, ratio of dilution water to weight and volume of organisms, and feeding schedule. Clean holding containers at least once every other day. If growths occur on sides of containers, dislodge and let settle for removal.

5. Parasites and Disease

a. *Stress in relation to parasites and infectious disease:* Unexpected and often unexplained mortalities in experimental and control animals interfere with acute or chronic test results. While many factors may be responsible for the death of an animal, diseases due to specific pathogens are among the most significant. In general obtain fish and other animals from specific pathogen-free stocks (commercial bioassay organism supplier, specific hatcheries, etc.) rather than stressing populations by parasite and disease controls. Also optimize laboratory conditions for the individual species to prevent the fostering of disease conditions.

When large numbers of organisms are retained in a relatively small space, undesirable growths, infectious diseases, and para-

† Some foods that have been used widely include Glenco Trout Food, Glenco, MN 53336; Biorell and TetraMin, available from local pet shops; Oregon Moist, Warrenton, OR 97146; and Cerophyl®, Agri-Tech, Inc., Kansas City, MO 64112. The latter has been used for the small forms and as a food for organisms providing food for the higher species.

§ Do not use acid and hypochlorite together.

sites may become a problem. Pathogens and parasites that might be very rare in natural waters become potential and ever-present dangers in intensive culture.

Filtration and/or sterilization of water, regular cleaning of holding vessels, strict sanitation practices, and sterilization of equipment are essential for healthy animals. Uniform food distribution, limiting the amount of unused food, and expeditious removal of unused food and waste materials are also important.

Organisms exposed to toxicants become stressed and weakened, and may become more susceptible to parasites and disease. Because other environmental factors contribute to reduced resistance, pay careful attention to nutrition, oxygen supply, and water quality.

b. Control methods: UV light and ozonation have been used successfully to control disease and parasites. Antibiotics used in holding tanks reduce bacterial populations. To reduce mortality and to avoid introduction of disease into stock tanks, treat with a wide-spectrum antibiotic immediately after collection, during transport, during egg production and hatching, or on arrival at the laboratory. Holding in a tetracycline-based antibiotic, || 15 mg/L for 24 to 48 h, may be helpful. Other chemotherapeutic agents are available, but use care in their application because some are toxic at low concentrations.²³ Do not use treated organisms for tests for at least 10 d after treatment unless eggs are treated and less than 48-h-old organisms are required for the test. If contamination is suspected, disinfect tanks and containers with 200 mg NaOCl/L for 1 h.

For larval tests, use strict sanitary measures including sterilization of utensils and containers, filtration and UV sterilization of water, and removal of metabolic products. If disease signs appear in larval cultures, discard the entire culture.

For tests using adult fish and shellfish, early diagnosis and prompt treatment, when available, can prevent losses.

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|| Terramycin or equivalent.

8010 F. Toxicity Test Systems, Materials, and Procedures

1. Water Supply Systems and Testing Equipment

a. Composition of materials used: Construct all components of a test system, including water heating and cooling units, constant-level troughs and head boxes, valves and fittings, diluters, pumps, mixing equipment, tanks, and exposure chambers, from inert materials. Acceptable materials include lead-free glass, perfluorocarbon plastics, silicone sealant and tubing, polyvinyl clear flexible plastic,* PVC, nylon, fiberglass, and No. 316 stainless steel. Unplasticized plastics, such as polyethylene or polypropylene, may be used in the dilution portion including holding and acclimation tanks. Avoid contact with brass, copper, lead, or rubber. Some neoprene formulations have been acutely toxic while others have been proven safe for toxicity testing. Cure materials in dilution water at least 48 h before use.

b. Temperature regulation: Obtain dilution water of the desired temperature by mixing hot and cold water of constant temperatures in the correct proportions, by heat exchangers, water baths, or by heaters or coolers in constant-level troughs and head boxes. A heated room or high-low incubator with thermostatic controls usually is suitable for static tests on warm-water organisms. Hold dilution water in tanks until it reaches ambient temperature for conducting static tests. For cold-water species use a specially insulated constant-temperature room or large water bath equipped with temperature controls and adequate circulating water. A satisfactory design for a small laboratory to conduct short-term static tests has been described.¹⁻³ Special facilities required for different groups of organisms are described in Sections 8111 through 8910.

c. Toxicant delivery system: In flow-through tests use metering pumps or other devices for accurate delivery of toxicant or test material into the dilution water.¹ Most toxicant delivery systems have been designed for fresh water and may not be applicable to all test substances. Deliver dilution water from constant-head troughs or head boxes by siphons, constricted tubing, nozzles, or pumps. Deliver toxicants by siphons from constant-head reservoirs, pumps, calibrated glass nozzles, solenoid valves, or Mariotte bottles.⁴ Mix dilution water and toxicant in tanks with baffles or stirrers or in mixing troughs.⁵ Since the introduction of the serial diluter,⁶ various methods and types of diluters have been described.^{1,7-25}

The choice of a toxicant-delivery system for flow-through toxicity tests depends on such factors as dilution, flow rate, quantity of toxicant available, and the presence of suspended solids. The proportional diluter¹⁷ was designed to handle a dilution factor (i.e., the factor by which a concentration is multiplied to calculate the next lower concentration) between 0.50 and 0.90. The serial diluter has been modified to provide a narrow range of concentrations.¹⁸ Other diluters operate well with a dilution factor of 0.50.^{1,23} Flow rates through test chambers may vary from 400 mL/min for the proportional diluter⁶ to 6000 mL/min.²³ Some stock solutions^{1,20,22-24} and other systems have been modified to handle larger volumes of toxicant and high suspended solids concentrations.^{1,20} These diluters are most

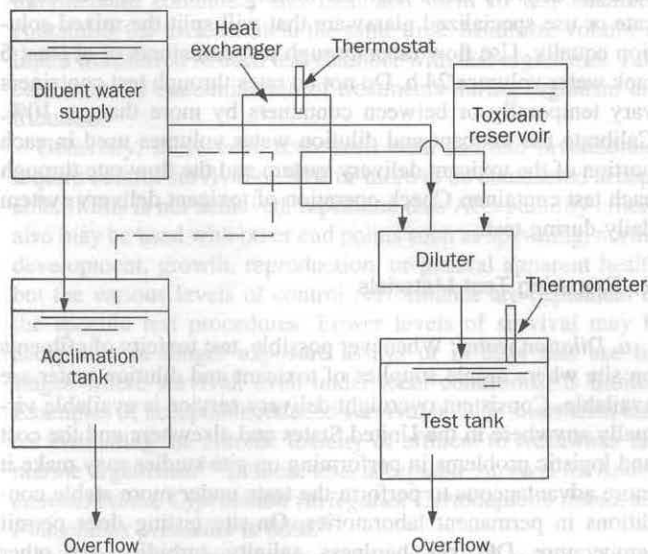


Figure 8010:3. Basic components of flow-through system.

suitable for effluent toxicity tests. Several diluters have been designed for flow-through toxicity tests with embryo-larval stages of aquatic organisms.^{1,22} One of these systems was designed to eliminate the air/water interface for tests using volatile compounds and compounds with very low solubility.²²

The basic components of a flow-through system are shown in Figure 8010:3. The diluent water reservoir is large enough to provide water for at least 5 d. If dilution water is added to this reservoir continuously, a smaller capacity is preferred. Dilution water flows at a constant rate by gravity from this reservoir to a constant-head diluent-supply head box through a nonmetallic float-controlled valve or other device and then to the diluter. Provide head box with heating or cooling equipment and a thermostat to maintain constant temperature. Equip test containers with an overflow system designed to prevent organisms from entering outlets. Clean test containers daily as described in 8010E.4d.²⁵

The constant-head toxicant supply is a constant-level tank, a Mariotte bottle, or other device. If toxicant is added at greater than a drip rate, adjust toxicant temperature to that of the diluter via a heat exchange; otherwise, the toxicant heat exchanger in Figure 8010:3 is not necessary. If the toxicant stock solution is unstable, renew it before it degrades. If metering pumps are used, the toxicant supply system need not maintain a constant head.

A simple valve control system for regulation of flow rates of dilution water and toxicant solution has been described.²⁶ For more toxic materials, less toxicant is required and a Mariotte bottle or syringe pump that delivers a very slow but constant flow is useful.^{27,28}

A diluter meters dilution water from the constant-head box and toxicant from the constant-head tank or other containers and mixes them in the proper proportions for each of the test chambers. After proper calibration of the diluter, make toxicant stock solutions to the proper concentration. Shield the toxicant supply reservoir from light when necessary.

*Tygon® or equivalent.

Provide a mixing chamber between diluter and test container for each concentration. If duplicate test containers are used, run separate delivery tubes from the mixing chamber to each duplicate or use specialized glassware that will split the mixed solution equally. Use flow rates through test containers of at least 6 tank water volumes/24 h. Do not let rates through test containers vary temporally or between containers by more than $\pm 10\%$. Calibrate the toxicant and dilution water volumes used in each portion of the toxicant delivery system and the flow rate through each test container. Check operation of toxicant delivery system daily during test.

2. Preparing Test Materials

a. Dilution water: Whenever possible, test toxicity of effluents on site where ample supplies of toxicant and dilution water are available. Consistent overnight delivery service is available virtually anywhere in the United States and elsewhere and the cost and logistic problems in performing on-site studies may make it more advantageous to perform the tests under more stable conditions in permanent laboratories. On-site testing does permit temperature, DO, pH, hardness, salinity, turbidity, and other qualities of the dilution water to vary normally with those of the receiving water.

Convey the effluent sample to testing chambers with as little modification as possible. Do not unnecessarily aerate, heat, cool, or agitate. In cases where the testing facility is remote from the effluent discharge site, artificial or reconstituted water may be used as the diluent. If the diluted effluent is low in DO, adjust flow-through and loading in the test chambers so that DO is not reduced significantly; aerate as a last resort. Hold temperature at or near that of the receiving water.

If the receiving water is deficient in DO and has temperatures above the locally applicable water quality standards, bring these into compliance so that allowable levels of a specific waste can be assessed meaningfully. Determine toxicity of test waste in conjunction with other contaminants present in receiving water by taking dilution water from receiving water just outside the area of effect of test waste. This is especially necessary when effluents contain metal salts, cyanide complexes, ammonium compounds, or other materials, the toxicity of which is greatly influenced by changes in pH, hardness, temperature, etc. If there are wide variations in receiving water quality characteristics, determine waste toxicity at the upper and lower limits of the range.

Evaluate receiving water effects on aquatic biota by using two controls, one with the receiving water and another (either natural or synthetic) with an unpolluted water of similar quality. If appropriate to the study, adjust calcium, magnesium, sulfate, alkalinity, pH, and DO for freshwater controls to those of the receiving water before adding wastes to determine if dilution water itself is unfavorable for the more sensitive aquatic species in the area. Conduct chronic tests to detect the most subtle sources of stress.

Turbidity of dilution water is important in determining harmful concentrations of potential toxicants because some toxicants are sorbed on particles. Turbid dilution water may limit visual inspection and photosynthesis of algae, affect the response of test organisms to tested material, form deposits, and clog water systems. When large amounts of settleable solids significantly

remove toxicants from the water, determine concentrations of toxicants in bottom sediments and their toxicity by appropriate tests with benthic organisms.

When the purpose of the test is other than to determine toxicity of an effluent, use for dilution water only a nonpolluted natural or synthetic dilution water of constant and reproducible quality that is favorable for aquatic life. Warm or cool dilution water to test temperature and bring to equilibrium with atmospheric gases (i.e., DO, CO₂) before use.

Use standard water conditions and organisms for comparative toxicity and sensitivity tests. Use reconstituted fresh or marine water [Sections 8010E.4b1) and 2)] if natural supply is not suitable. Because of the effects of water quality on toxicity, it may be desirable to use both hard and soft water for tests on freshwater organisms.

Many marine organisms spend a portion of their life cycles in estuaries. In life-cycle tests, change dilution water in accordance with their requirements at different life stages. If the effects of temperature are not being studied, keep it within a favorable range.

For warm-water ($\geq 20^\circ\text{C}$) species, keep DO greater than 40% of saturation; for cold-water species, keep DO greater than 60% of saturation. Some larval forms, such as those of marine crustaceans, may require higher DO concentrations.

Determine pH requirements for test organisms. In long-term studies, other than effluent and monitoring tests, keep pH within 0.4 units of the desired value. Avoid rapid changes in temperature, pH, or CO₂ content. A rapid increase in the CO₂ content of marine waters indicates that some significant change has occurred that should be investigated at once. Freshwater organisms are more tolerant of pH changes and accommodate to much wider variations than strictly marine forms. Changes in pH drastically alter toxicity of many materials, for example, cyanide and NH₃.

In working with estuarine and marine organisms and different life stages that may be marine or estuarine, salinity is of prime importance. Use the natural salinity for each test species and its different developmental stages.²⁹

Keep acidity, total alkalinity, and hardness of dilution water constant. Alkalinity and hardness influence toxicity of some metals and total alkalinity is an important factor in photosynthesis and algal growth.

b. Toxicant solution: Prepare toxicant solution in advance and add immediately to the dilution water for static tests. If a toxicant is unstable, determine its stability and replace as necessary. If possible, measure toxicant concentrations during the test. Prepare all solutions for each series of tests from the same source sample. Disperse undissolved material uniformly by shaking or gently mixing.

If solvents are necessary, use acetone, dimethylformamide (DMF), ethanol, methanol, isopropanol, acetonitrile, dimethylacetamide, ethylene glycol, or triethylene glycol to prepare stock solutions. Certain surfactants may be useful. Use only the minimal amount of solvent necessary to disperse the toxicant. Do not exceed 0.5 mg/L in static and 0.1 mg/L in flow-through test solutions. A solvent control is also necessary when a carrier is used.

If a solvent is used, use two sets of controls, one containing no solvent and the other containing the highest concentration of solvent used during the test.

Some effluents, especially oily wastes, are difficult to distribute evenly. The nature of the test material (e.g., single chemical or solid versus effluent) governs the preparation of test concentrations and frequency of test medium replacement. Common problems include insolubility, adsorption on exposed surfaces, decomposition, photolysis, loss of volatiles, high BOD and/or COD levels, and bacterial growth. These can change the apparent test concentration and lead to erroneous results. Effluent samples that vary in composition with time may require a series of tests to characterize toxicity.

Store effluent samples, in containers from which all the air has been expelled, at $4 \pm 1^\circ\text{C}$. Do not store samples longer than absolutely necessary because toxicity may change with time. (Choose sample containers to minimize changes in concentration of constituents in sample.)

Thoroughly mix test materials before use. Use material directly as a stock solution of toxicant or prepare a stock solution using filtered dilution water. Make stock solutions with dilution water on a volume-to-volume basis. If the effluent is liquid, designate the percentage waste in each test concentration. If the waste is a solid, dilute on a weight-to-volume basis, e.g., milligrams per liter.

If the waste contains both solids and liquids, mix thoroughly to disperse before using as a stock toxicant, and provide agitation in the stock reservoir and test containers. If larger organisms are tested, a propeller placed under a screen or perforated false bottom may be used to maintain consistency of the solution. If the solids settle out rapidly and do not contact pelagic organisms, test only the liquid portion. After thorough mixing, let settle and decant or drain off the liquid for use as test toxicant. If the solid waste portion is toxic, set up test chambers having a certain weight-to-volume ratio of bottom material and expose benthic and burrowing organisms of the receiving water area. Mix wastes and let settle before adding organisms. If waste contains sparingly water-soluble materials check solubility and if below or at very low toxic concentrations, solvents, emulsifying agents, or water-miscible solvents may be used to disperse.

c. Test organisms: Select test organisms as described in Section 8010E.1 and handle as indicated in Sections 8010E.3 and 4. For long-term tests, use only the highest quality test organisms available. At the end of the test, control organisms still should be in good condition.

3. Test Procedures

a. Experimental design: Expose test organisms in at least duplicate containers of each experimental concentration. By using more organisms and replicate test containers for each toxicant concentration, intratest variability can be evaluated. Individual test methods may specify the number of replicates. Statistical methods also may dictate numbers of replicates. Use only true replicates with no water connection between test containers. Typically, each test consists of a minimum of five test concentrations and a control, with an additional control if a solvent is used. Tests of ambient waters may make the typical dose-response test design impractical. To compensate for positional effects, arrange test containers at random in the testing area. If replicates are used, randomize each series of test containers separately. Distribute organisms randomly to test containers either adding one at a time to each container if there are

to be less than 11 organisms per container or two at a time if there are to be more. In short-term static tests, add organisms to intermediate containers and then add them to test chambers containing the toxicant all at the same time. Minimize volume of liquid transferred to each test chamber with test organisms. Take care to avoid contamination of treatments during organism distribution.

Generally, short-term, acute tests with fish and invertebrates require control survival of 90% or more to be considered acceptable. If this is not achieved, repeat the test. Acceptability criteria also may be used with other end points such as spawning, normal development, growth, reproduction, or general apparent health, but the various levels of control performance are dependent on the specific test procedures. Lower levels of survival may be acceptable in longer exposure assays or in tests that use life stages where survival, even under ideal conditions, is limited. Examples of acceptable reduced survival include short-term tests for estimating the chronic toxicity of effluent to freshwater and marine organisms.^{2,3} In these tests acceptable survival for *Americamysis bahia*, *Cyprinodon variegatus*, *Ceriodaphnia dubia*, and *Pimephales promelas* is 80%.^{2,3}

In short-term static or renewal tests with fish, use 20 or more test organisms in each toxicant concentration. Use of larger numbers of organisms per test concentration for smaller organisms is desirable. The number of organisms exposed in each test concentration is governed by size of the organism; expected normal mortality; extent of cannibalism; availability of dilution water, toxicant, and test organisms; and desired test precision. Test precision depends on variability of organism response, number of organisms exposed to each concentration, number of replications, differences between concentrations and range of concentrations tested, and toxicant concentration and its variability.

For a given test increasing the number of test organisms increases confidence in estimates of effect and precision. It is recommended that the 95% confidence interval be less than $\pm 30\%$ of the mean. With test organisms for which culture methods are not available, this precision may be difficult or impossible to attain. Reference toxicant tests are used to evaluate the condition of the test organisms and may be used to evaluate intertest performance of the method.¹⁻³ Make specifically designed intra- or inter-laboratory tests to evaluate precision.

Determine length and weight of representative organisms before the test to establish loading rates and acceptability with respect to size variation. After acclimation has begun, handle test organisms as little as possible. Increases in weight or growth may be determined by adding more animals than required initially so that some may be removed to make necessary measurements. At the end of the test, measure weight and length to determine sublethal impacts for computation of statistical end points (see 8010G).

b. Selecting test concentrations: Express liquid waste concentrations as percent on a volume-to-volume basis. Express concentrations of nonaqueous wastes and of individual chemicals as milligrams or micrograms per liter. Clearly indicate what the weight represents as inclusion of water of hydration is part of the weight of the solute (e.g., $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$). It often is more appropriate to express weight as weight of toxicant (e.g., $\mu\text{g Cu/L}$). When an impure chemical is tested, especially in a formulation containing added inert ingredients, indicate the chemical com-

TABLE 8010:VI. PERCENTAGE OF AMMONIA UN-IONIZED IN DISTILLED WATER*

Temperature °C	Percentage Un-ionized at Given pH									
	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	
5	0.01	0.04	0.11	0.40	1.1	3.6	10	27	54	
10	0.02	0.06	0.18	0.57	1.8	5.4	15	36	64	
15	0.03	0.08	0.26	0.83	2.6	7.7	21	45	72	
20	0.04	0.12	0.37	1.2	3.7	11.7	28	55	80	
25	0.05	0.17	0.51	1.7	5.1	14	35	63	84	
30	0.07	0.23	0.70	2.3	7.0	19	43	70	88	

* Prepared from data given in Sillen and Martell.³⁰

position by weight and whether the EC value is based on concentration of total material or active ingredient.

Although test end points such as EC or LOEC may be determined by using any appropriate series of test concentrations, the geometric series of concentration values is simplest to use when the approximate toxicity range is unknown. Multiply the highest and succeeding concentrations by a constant factor (0.3 to 0.5) to obtain concentrations that are evenly spaced on a logarithmic scale. Range-finding tests can help determine the dilution factor to use in subsequent tests.

The magnitude of concentration intervals to establish an EC by interpolation depends on the required degree of confidence in the point estimate and on the experimental data. Intervals spaced more tightly around the expected EC will give a more precise estimate of the true EC.

c. Loading: For static tests do not exceed an organism loading of 0.8 g/L in the test container. In tests with small organisms and tropical forms, decrease loading to as low as 0.1 g/L and accommodate large test organisms by using larger or duplicate test containers. Limit the number of test organisms per volume of test solution so that during the test (a) DO remains greater than 60% of saturation for cold-water species and greater than 40% of saturation for warm-water species; (b) toxicant concentration is not lowered significantly; (c) concentrations of metabolic products (e.g., NH_3 , CO_2) do not become too high; and (d) organisms are not stressed by crowding or increased potential for cannibalism. Do not let concentration of un-ionized ammonia exceed 20 $\mu\text{g NH}_3\text{-N/L}$ (Table 8010:VI).

For flow-through studies, use a flow rate of at least 6 tank volumes/24 h to maintain desirable temperature and DO and safe concentrations of metabolites. If the DO concentration drops below the desired level, increase the turnover rate within the diluter. If this is inadequate, aeration of the test chambers may be permissible.

d. Physical and chemical determinations:

1) Dilution water analysis—For fresh water, measure hardness, alkalinity, pH, TOC (or COD), and suspended solids at least once every 30 d and at the beginning and end of the test. If water quality is variable, test more frequently. Tap water can be dechlorinated by active aeration (using air stones) for 24 h, filtration through activated carbon, or use of sodium thiosulfate. If a treated tap water is used, measure residual chlorine by one of the methods given in Section 4500-Cl.³¹

Analyze weekly for pH, alkalinity, and hardness to define test-water variability. If characteristics are affected by the toxicant,

test samples from each toxic concentration at least once every other week. For brackish or marine dilution water, measure salinity, pH, DO, and temperature two or three times daily; and suspended solids and TOC at least once every 30 d and at the beginning and end of each test.

2) Toxicant analysis—For flow-through life-cycle tests it may not be necessary to make routine detailed analyses, but make periodic tests to insure that the correct ratio of effluent to dilution water is maintained in exposure tanks.

For studies to determine chemical water quality criteria, it is desirable to measure concentration of toxicants in each container at beginning and at least once during the test or weekly in longer tests. Chemical measurements should be made in at least one container at the next-to-lowest toxicant concentration (or within the calibration range of analytical method). Whenever a malfunction is detected in any part of toxicant delivery system, check toxicant concentration in at least one container. For replicate test containers use a ratio of highest measured concentration to lowest measured concentration of less than 1.15; if this is exceeded, check toxicant delivery system and analyze additional samples from test containers to determine if the sampling or analytical method is sufficiently precise. Do not accept measured toxicant concentrations differing by more than $\pm 15\%$ from the calculated concentration unless specific reasons justify a greater difference. Precision of chemical test methods is important for assessing measurements of nominal toxicant concentrations, and test methods ideally should be calibrated in a range that includes the nominal toxicant concentration.

Record temperature at least hourly throughout the test (24 h/d) in at least one test container and make additional measurements on dilution water and other test solutions. If test is performed in a laboratory, continuous monitoring of temperature of the testing area (i.e., water bath, environmental chamber, etc.) may be appropriate. Measure DO, pH, and salinity at the beginning of the test and daily thereafter in the control, high, medium, and low toxicant concentrations. Generally, variation should not exceed $\pm 1.0^\circ\text{C}$.

Take water samples for chemical analysis at the center of the exposure tank; do not include surface scum or material from tank bottom or sides. If analytical results are not affected by storage, collect daily, equal-volume grab samples and composite for a week. Analyze sufficient samples throughout the test to determine whether the concentration of toxicant is reasonably constant. If it is not, analyze enough samples weekly to show the variability of toxicant concentration. If methods are available, determine in the next-to-lowest concentration the loss of toxicant. If the loss is more than 10%, attempt to alleviate by using either a faster flow rate or a lower loading.

When necessary, analyze mature and immature test organisms for toxicant residues. For larger organisms analyze muscle and liver and possibly gills, blood, brain, bone, kidney, GI tract, gonads, and skin. For large organisms, analysis of whole specimens can be used but does not replace analysis of individual tissues, especially muscle (edible fillet).

e. Biological data and observations: In short-term tests with macroinvertebrates and fish, count number of dead or affected organisms in each container at least daily throughout the test. With certain fast-acting biocides it may be useful to count number of dead or affected organisms in each container at 1.5, 3, 6, 12, and 24 h after beginning the test. Remove dead organisms

as soon as observed. Often it is more important to obtain data that will define the shape of the toxicity curve than to obtain data at prespecified times.

Death is the adverse effect most often used to reflect acute toxicity. The usual criterion for death is no movement, especially no gill movement in fish, and no reaction to gentle prodding. Death is not easily determined for some invertebrates. Cessation of movement of antennae, mouth parts, or other organs may be used. When death cannot be determined, use EC50 rather than LC50. The effect usually used for determining EC50 with daphnids, midge larvae, copepods, and other organisms is immobilization, defined as inability to move, except for minor activity of appendages. Other effects can be used to determine EC50, but always report the effect and its definition. Consistency in defining the effect directly influences toxicity test precision and repeatability. Also report such effects as erratic swimming, loss of reflex, discoloration, changes in behavior, excessive mucus production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism.

In short-term tests, organism reactions during the first few hours may indicate the nature of the toxicant and serve as a guide for further tests.

In long-term partial- or full-life-cycle tests a photographic method for counting and measuring small test organisms has proven useful.³² This is rapid and accurate, and does not entail handling the organism. With this method, use exposure tanks with glass bottoms and drains that allow the water level to be drawn down. To count and measure test organisms, draw water down to a depth of 2 to 3 cm and transfer tank to a light box having fluorescent lights under a square millimeter grid of adequate size. Photograph aquarium bottom; this shows organisms over the grid. On an enlargement of the picture, count and measure organisms.

f. Photoperiod and artificial light: In long-term studies to determine water quality requirements for those species requiring annual-light-cycle photoperiods, simulate natural seasonal daylight and darkness periods at the locality or some central location.³³ (See Section 8910 for more information on light cycles for fish.)

Use cool white fluorescent tubes or a wide-spectrum lamp as a light source.[†] Some organisms require subdued light, others need a place to hide, and some, such as lake trout eggs, require darkness during certain life stages. Base exposure to light on what is normal to, and required by, the species. Measure light intensity at the water surface. In short-term tests a standard photoperiod of 16 h light, 8 h dark is suggested.

g. Exposure chambers: For organisms weighing more than 0.5 g, use a test solution between 15 and 30 cm deep. In short-term tests, these organisms often are exposed in about 15 L solution in 20-L wide-mouth, soft-glass bottles. Fabricate test containers of other sizes by welding (not soldering) stainless steel, by gluing double-strength or stronger window glass with clear silicone adhesive formulated for aquarium use, or by modifying glass bottles, battery jars, or beakers to provide screened overflow holes or V-notches. Because silicone adhesives absorb some organochlorine and organophosphorus pesticides, expose

as little of the adhesive as possible to the water. Place extra beads of adhesive for added strength only on the outside of containers. Expose smaller organisms in 30-mL to 2-L beakers that contain 15 to 1500 mL solution. Expose daphnids, midge larvae, copepods, and other small organisms in loosely covered beakers or other containers. Disposable plastic containers may be used for tests involving compounds that do not react with the plastic. Disposable plastic containers are recommended for such tests as the 7-d *Ceriodaphnia* survival and reproduction assay³ and *Champia parvula* sexual reproduction assay.²

With flow-through tests keep liquid surface area/volume ratio small to reduce loss of volatiles. For various exposure chamber designs see Sections 8211 through 8910.

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[†] For example, OPTIMA 50, Duro-Test Corp., North Bergen, NJ 07047, or equivalent.

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8010 G. Calculating, Analyzing, and Reporting Results of Toxicity Tests

This section identifies statistical methods used in analyzing data from acute and chronic toxicity tests, evaluates methods appropriate for each type of data, and discusses advantages and disadvantages of the statistical methods that are used most often. If unfamiliar with basic statistical concepts and methods, consult a statistician when questions concerning data analysis arise.

The precision of a biological test is limited by a number of factors including the normal biological variation among individuals of a species, ruggedness of test protocols, and analyst proficiency. Calculation of test end points from experimental data therefore will reflect the net effect of these test variables.¹ In general, the statistical methods presented in this section are applicable to both acute and chronic methods. Traditionally, however, some techniques have been used specifically for acute test results, and others for chronic test results.

1. Toxicity Data Analysis

Data generated in acute toxicity tests are quantal, that is, responses are measured with yes/no-type observations (e.g., did

exposure cause immobilization, death, or not?). Continuous measurements that are measured in quantitative or graded tests, such as length, weight, or number of young produced, usually are not utilized as end points in an acute toxicity test. Data generated in chronic toxicity tests may be quantal (e.g., long-term survival), continuous (e.g., growth), or count (e.g., number of young produced).

Results from chronic toxicity tests may be analyzed by one or more statistical methods, depending on the purpose of the test. Regression methods, both parametric and nonparametric, may be used to generate ECs, evaluate conventional concentration-response test designs, and provide confidence intervals for point estimates (e.g., EC50 or LC50) interpolated from the appropriate model. Hypothesis test methods (both null and alternate) can be used to generate estimates of effect threshold (e.g., NOEC, LOEC) or estimates of equivalence (e.g., "bioequivalence"). The advantages and drawbacks of these different statistical applications have been reviewed in detail.^{2,3} All of the methods are computationally intense, but availability of computer software

applications and descriptive material facilitate their use in most instances. Although more than one statistical method can be used to treat a given data set, the experimental design chosen for the bioassay may affect the robustness of the statistical results. For example, point estimates and confidence intervals derived from regression models benefit from designs with more doses near the effect level of interest, while hypothesis test methods improve (in terms of statistical "power") with an increase in number of replicates at tested doses. In order to estimate an effect (or the test concentration below a specified effect) regression methods interpolate points on the modelled concentration-response curve, whereas the hypothesis test results are constrained to be one of the doses tested. In this sense, both regression and hypothesis test methods benefit from having the selected test concentrations bracket close to the "true" effect level of interest. If the purpose of testing is to compare sources or evaluate changes in toxicity over time, then effect levels from appropriate regression methods are preferred because the results from multiple tests are amenable to normal population statistics, whereas multiple NOECs are not.

ECP values can be calculated from parametric models such as probit, logit, and GLiM.⁴⁻⁶ Choose the model appropriately for the type of data generated in the test. For example, probit analysis is only suitable for quantal data,⁷ whereas the GLiM models require the appropriate data link.⁵ The nonparametric ICP or linear interpolation model is also available for evaluation of nonquantal data.⁸ Care must be given to evaluating goodness of fit and biological plausibility of the resultant toxicity curves and confidence interval data with any model results. The model should be able to account for apparent thresholds or low dose enhancement (nutritive or hormetic effects) in test data. Models that "force" data to fit by averaging adjacent dose response data ("smoothing") or extrapolating to zero may not be appropriate for use on a given data set.

The NOEC (no-observed-effect concentration) or LOEC (lowest-observed-effect concentration) values calculated from hypothesis test statistics are based on statistical significance (usually at the $P = 0.05$ level): the NOEC is the highest dose tested with a response not statistically different from control response, and the LOEC is the lowest dose tested having a statistically significant difference from control response. The NOEC can be used for analysis of any biological end point and data type commonly encountered in aquatic toxicity testing. The Chronic Value, ChV (sometimes referred to as MATC) is a point estimate determined as the geometric mean of the LOEC and NOEC doses. Hypothesis test results are sensitive to intratest variability which controls statistical power, and results must be examined for homogeneity in variance among test groups as well. Data transformations usually are required (see ¶ 2 below).

Recently, the alternative hypothesis method (sometimes termed "bioequivalence") has been proposed and discussed^{3,9} as an adjunct method for comparing the toxic response of a specified sample concentration to a threshold or acceptable level of response. This method has application where it is desirable to determine statistically that a specific sample response is equivalent to, rather than different from, a targeted value. It is useful in pharmacokinetic applications or other experimental designs where limited numbers of doses can be tested, and when emphasis on the impacts of false-negative rather than false-positive statistical errors is important. In simplest form it is applied in a

two-concentration test design (control and one dose, or two different doses), and is subject to the same concerns over variance and power as the null test.

Either hypothesis test method can be used to compare statistical significance between point estimates derived from regression models. This may be useful for comparing different species' response to toxicants, or different temporal responses.

a. Calculation of LC50: Acute toxicity test results generally are characterized by the median lethal concentration, LC50, when mortality is the end point or median effective concentration, EC50, when a sublethal effect is the end point. The LC50 is an estimate of the true median lethal concentration of the test material for the entire test species. Therefore, also provide a measure of statistical confidence in the point estimate, such as the 95% confidence interval of the LC50; values other than 50% can be used to characterize toxicity; however, the precision of test results for a typical sigmoid (or "s"-shaped) cumulative distribution dose-response curve may be best in the vicinity of the 50% effect level because this is the straightest part of the curve. LC values near the tails of this curve (e.g., LC10 or LC90) have wider confidence intervals.

Numerous procedures are available for analyzing quantal toxicity data. LC50 calculations include parametric procedures such as probit analysis,^{7,10} logit,¹¹ and generalized linear models (GLiM).^{4,5} The most commonly used nonparametric procedures are the Spearman-Kärber method and the trimmed Spearman-Kärber method,¹² while numerical interpolation techniques include graphical interpolation, moving average interpolation, and the binomial distribution. No single method is most appropriate for all data sets, but graphical interpolation and binomial distribution methods are simple to use. Personal computers facilitate the use of more sophisticated statistical methods and models, which may provide better fit of the experimental data for many data sets. The statistical procedure cannot always be selected before the test is conducted because the data generated must meet certain model assumptions or minimum criteria before the applicability of various methods can be determined.

Parametric procedures transform dose-response data to a known or expected functional form before the LC50 determination (parametric methods). The probit method¹⁰ probably is the most widely used LC50 calculation procedure and uses the probit transformation of mortality data in combination with a standard curve-fitting technique. A second parametric procedure utilizes a logit transformation¹¹ of mortality data. Common disadvantages of the parametric computational methods are that the distribution properties data must meet the model assumptions or the LC50 produced will not be considered appropriate. The probit and logit methods yield symmetric dose-response curves; they are not valid if the true curve is asymmetric. Models (GLiM) have been used to avoid such drawbacks.^{4,5} Additionally, unless 0 and 100% mortality data are adjusted, the parametric methods can be utilized only when at least two partial kills are present in the data set.

The nonparametric Spearman-Kärber method and the trimmed Spearman-Kärber method¹² do not require that data meet model assumptions and do not depend on the presence of partial kills. These methods estimate the LC50 if the dose-response curve is symmetric. However, if the true curve is asymmetric, the Spearman-Kärber method estimates the mean of tolerances and the trimmed Spearman-Kärber method estimates the trimmed mean

of tolerances; the LC50 is the median of tolerances. One drawback of the Spearman-Kärber method is that the test data must cover the range from 0 to 100% mortality.

The moving average and moving average angle (transformation of mortality to angular values) methods also do not assume that the data distribution fits a predetermined model nor require partial kills for the LC50 calculation.¹³ Shortcomings of these two methods are that the concentration series of the test must be equally spaced, and the methods cannot be used to calculate an LC value other than the LC50. Median effective time (ET50) for mortality at each concentration is estimated by plotting percentage mortality on a probit scale against time on a logarithmic scale and then using probit analysis techniques similar to those given above.^{10,14-16} Procedures are the same, although more frequent observations of mortality may be required.

Single chemical toxicity tests often use preliminary range-finding tests with a wide range of test concentrations to obtain an estimate of toxicity. For subsequent definitive tests, a dilution series with more tightly spaced concentrations is used. For effluent toxicity tests, range-finding tests may not be feasible, because of stipulated limitations on effluent sample holding times. In these instances, effluent tests often produce results in which all organisms live at one test concentration, while all organisms die at the next higher concentration (i.e., no partial kills). For this type of data, the binomial test provides an approximate LC50 and specifies the concentration that can serve as statistically sound 95% confidence limits. However, the binomial test does not provide a true LC50 estimate and should be used only when the other available methods (e.g., probit) cannot be used. A preferred alternative to the binomial distribution method is the Williams test.¹⁷

Manual techniques for graphical determination of LC50 and confidence limits are available, and have been described in past editions of *Standard Methods*. A number of user-friendly computer software packages,* which can be used for parametric and nonparametric methods for treatment of data and generation of confidence limits, also are available. Because each of these methods has limitations with respect to certain data characteristics that can be analyzed (i.e., concentration series, number of partial kills, data type, data distribution, etc.) take care in using the appropriate method with the experimental data.

b. Control mortality: Generally, control mortality should not be greater than 10%. More than this is usually unsatisfactory and requires repetition of the test. Sometimes with long-term tests or with some invertebrates that have considerable mortality under the best possible conditions, it is necessary to use Abbott's formula:¹⁸

$$P = \frac{P^* - C}{1 - C}$$

where:

P and P^* = corrected and observed proportions responding to the experimental stimulus and
 C = proportion responding in the control.

This approach does not solve the problem of possible interaction effects of the toxicant with whatever is causing mortality in the control.

Stephan¹⁹ reviewed the methods available for calculating LC50s and recommended use of the moving average method and log concentration when one or more partial kills are present in a data set, and use of either binomial or moving average when no partial mortality is observed. Data sets of the latter type are not desirable when accurate estimates of toxicity are required.

A review of LC50 calculation methods²⁰ concluded that if the effect data are normally distributed, then probit was the most efficient method to use for quantal data, whereas the trimmed Spearman-Kärber method was preferred when normality did not hold. While statistical models other than these have been recommended and used^{5,8} for continuous and count data, similar evaluations of how the data fit the model assumptions must be made for any regression model. Because there may not be any single LC50 technique that is always best in all situations, goodness-of-fit estimates of the model outputs, as well as confidence limits, must be evaluated.

c. Hypothesis test methods for analyzing toxicity data: Traditionally, these methods have been used for chronic toxicity test results rather than for acute test results. However, with development of short-term critical life-stage test methods, many of which have lethality end points, the distinction between acute and chronic is sometimes specious; thus these statistical methods need not be limited to one type of toxicity test. Determine NOECs and LOECs with available computerized statistical methods for hypothesis testing (Dunnett's Test, Bonferroni's T-Test, Steel's Many-One Ranked Test, or Wilcoxon Rank-Sum Test.²¹ Parametric statistical methods require that individual test data meet normality assumptions, and that the variances of different treatment groups within a test are homogeneous. Therefore, before using these methods check the data to ensure that model assumptions are met. Use the Chi-square or Shapiro-Wilk's tests to test for normality, and Bartlett's test for homogeneity of variance. Statistical software packages (§ 1a above) are suitable for these procedures.

Often it is necessary to transform data to conduct desired statistical analyses. Perform certain transformations routinely before any analysis. One example is percentage data (e.g., percent fertilized, hatch, survival). Adjust percent or proportion data by using the arcsine square-root transformation before using Dunnett's Test. This transformation corrects for the bias in treatment group variances that is expected for percent data. Transformation (e.g., log, square root) of quantitative data such as number of young or larval weight sometimes is useful in helping the data meet the assumptions of normality and homogeneity of variance.

d. Regression models for point estimates: ECP values other than median lethal concentrations are frequently desirable, particularly in chronic tests. Point estimation techniques for LC, EC, and IC determinations include probit, logit, GLiM, and linear interpolation. Linear interpolation and probit methods are available in the software packages referenced in § 1a. GLiM models also are available in software products.† Probit usually is con-

* Such as TOXSTAT®, University of Wyoming, Laramie; TOXCAL®, Tidepool Scientific Software, P.O. Box 2203, McKinleyville, CA; Toxdat, Statistical Support Staff, Biological Methods Branch, Environmental Monitoring and Support Laboratory, U.S. EPA, Cincinnati, OH.

† Such as SAS®, SAS Institute, Cary, NC; The GLiM System, Release 3.77, Numerical Algorithms Group, Oxford, UK; or S-Plus®, Statistical Sciences, Seattle, WA.

sidered to be effective near median effect levels, and should be used only for quantal data; check to see that normality assumptions are met. Nonmonotonic dose-response curves, especially those with low-dose enhancement or thresholds, may best be analyzed by the appropriate GLiM model with data link,^{4,5} or the nonparametric linear interpolation method.⁸ The latter is often the most efficient method for calculating point estimates from nonquantal test results. One of the advantages of using an appropriate regression model is that nominal confidence intervals can be calculated for any given point estimate. This information can be used to help evaluate fit from alternative models, and, more importantly, provides an expression of statistical confidence in the test result. Confidence intervals can be constructed from the entire data set, or just the means of the experimental replicates. Typically, expect better coverage of the nominal confidence interval range from the former. "Bootstrapping" techniques typically are used for constructing confidence intervals, but the user should refer to the specific software provider or available literature for more information on specific methods.

e. Plotting the data: Many anomalies and trends in test results are not obvious unless the data are plotted. Plot chronic toxicity test data before making statistical analysis to assist in proper interpretation of results. By plotting the data, answers can be obtained to important questions, including the following: (a) Is the variability among replicates homogeneous across all concentrations? (b) What is the pattern of response vs. concentration—is the dose-response curve monotonic? (c) Do some concentrations have enhanced response relative to controls? (d) Do some data appear to be highly unusual (outliers)? (e) Is the effect at the LOEC consistent with the concentrations above and below it, or could it be different just by chance? and (f) Do the confidence limits look plausible for the shape of the curve?

f. Outliers: Outliers are data points that are inconsistent with the trends exhibited by the majority of the data. They are detected most often by visual examination of plots of the data. Standard procedures for statistically detecting outliers are available²¹ but should be used with extreme caution. In many instances, outliers are a result of measurement error, transcription, or data entry error. Because apparent outliers are usually mean values computed from replicates, inspection may show that the mean was affected by just one replicate, or that the variance among replicates was quite large. If the error can be traced and be corrected justifiably, then it is acceptable to do so. If not, make the analysis with and without the data point in question and report the results of both analyses. Regression methods usually are more robust to the effects of an outlier than are hypothesis test methods, and thus are a more critical concern in the latter.

2. Reporting Results

Report results from toxicity tests as completely as possible so that any conclusions can be evaluated independently. Include all of the following that are applicable: (a) test organisms used, including species, age, life stage, food used in cultures, acclimation, mean length, and weight, reference toxicant test data for the test population, diseases and treatment, source, and observations on behavior during test; (b) tested material: its source, storage, physical and chemical characteristics, and collection method and time; (c) dilution water: its source, storage, physical and chem-

ical characteristics, collection method and time, pretreatments, additives, preparation (if applicable) and known contaminants; (d) test solution: its physical and chemical properties, especially toxicant concentrations (if applicable), and temperature; (e) test method, end point(s) of test, deviations from referenced procedures, data and time of initiation and termination, type and volume of test chambers, volume of test solutions, number of replicate test chambers per treatment, number of organisms per replicate, toxicant delivery, system, and flow rate or frequency of renewal; and (f) quality assurance methods used to ensure data integrity.

Present raw data for individual biological end points (e.g., mortality) and water quality measurements. Reference statistical methods and provide tabular summaries on toxic end points (e.g., LC50 with confidence limits, LOEC, NOEC, chronic value) and physical and chemical data. If applicable (e.g., effluent testing), include quality assurance data, such as results of reference toxicant tests, in tabular form.

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8010 H. Interpreting and Applying Results of Toxicity Tests

1. Interpretation of Results

The 48- and 96-h LC50 values produced from standard acute toxicity tests are useful estimates of relative acute lethal toxicity to test organisms under specified conditions. Without additional data, however, these values do not necessarily have any direct meaning in terms of "safe" or "hazardous" conditions in natural water (e.g., exposure analysis). Long-term exposure to much lower concentrations may be lethal to fish and other organisms and/or may cause nonlethal impairment of their function. Similarly, short-term exposure to these or higher values of total contaminants may cause no discernible effect. Numerous site-specific factors may influence the effect of the test material on the biota of a receiving water body.^{1,2}

2. Influence of Test Conditions

The results obtained in a toxicity test, in large part, depend on the conditions and nature of exposure; they are the product of operationally defined procedures. Therefore, it is important to select the testing procedures carefully to provide appropriate conditions and ensure that the results are applicable to the water quality problem at hand. At the outset define the problem carefully and succinctly and establish how the results of the toxicity test will assist in the problem solution. Selection of type and species of test organism, life stage, test response, duration of test, and physical and chemical conditions of test are key factors in obtaining useful, interpretable toxicity test results.

Although not always possible, it usually is desirable to prescribe toxicity test conditions that are as close as possible to the natural environmental conditions. For some variables such as pH, temperature, and contaminants, even small differences be-

tween the laboratory test conditions and those in the natural environment can affect substantially, and therefore reduce, the utility of the results. Some situations dictate that "standard" toxicity tests (i.e., incorporating a standard set of test conditions such as 96-h exposure, standard chemical and physical conditions, and standard test organisms) be run in addition to those incorporating conditions more similar to those of the environment of concern. This practice provides results that could be compared with those reported in the literature as well as a potential route for adapting or interpreting results of other standard toxicity tests. In some instances, in-stream toxicity tests with caged organisms or laboratory tests using receiving-stream waters are a more reliable means by which to evaluate ambient toxicity. Such procedures can test for the impact of a complex variety of contaminants.

In the interpretation of toxicity test results to predict in-stream effects,³ consider such factors as expected rate of dilution, aquatic chemistry, bioavailability, and duration and pattern of organism exposure in the environment. In addition, consider the function and sensitivity of the test organisms, compared to resident species of concern. Apply hazard assessment approach⁴ to interpret more accurately the impact on aquatic organisms or designated beneficial uses of the water.

In recent years, aquatic toxicity testing has been applied to a variety of different regulatory and scientific purposes, including toxicity testing of municipal and industrial effluents as part of monitoring/permit compliance,^{2-3,5-7} the derivation of national and site-specific water quality criteria for individual chemicals,^{1,8,9} product safety evaluations,¹⁰ chemical persistence studies,^{5,11} effluent interaction studies, testing of leachates and sediments, and studies included in Toxicity Reduction Evaluation (TRE) programs to identify constituents causing toxicity in ef-

fluents.² These diverse applications have broadened the utility of toxicity testing, and made more important the judicious interpretation of their results.

3. Statistical Interpretation

It is crucial in conducting or interpreting the results of a toxicity test to understand clearly that statistically significant differences between control and test organisms, upstream and downstream populations, seasonal variations, etc., are not necessarily changes or differences that have ecological impact. Conversely, trends or other changes that appear to have biological/beneficial use significance may not be statistically demonstrable because of sample size or other limitations.

While the application and interpretation of toxicity tests in water quality management programs may appear to be more difficult than the more frequently used chemical test, they offer certain advantages: they directly address bioavailability and the complex interaction of multiple chemicals, they yield a single integrated measurement of organism response to a chemically complex sample, and they may be less expensive and easier to interpret than a series of chemical measurements.

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8010 I. Selected Toxicological Literature

Toxicity testing has become an integral part of the evaluation of the effects of waste discharges on the aquatic environment. Listed below are the principal scientific and engineering journals publishing results of original toxicological research. Textbooks and manuals that summarize and synthesize the results of original research also are listed.

1. Journals

Aquatic Toxicology
Archives of Experimental Contamination and Toxicology
Archives of Toxicology
Bulletin of Environmental Contamination and Toxicology
Chemosphere
Ecotoxicology and Environmental Health

Environmental Pollution
Environmental Toxicology and Chemistry
Marine Environmental Research
Marine Pollution Bulletin

2. Textbooks and Manuals

BURTON, G.A., JR. 1992. Sediment Toxicity Assessment. Lewis Publ., Boca Raton, Fla.
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8020 QUALITY ASSURANCE AND QUALITY CONTROL IN LABORATORY TOXICITY TESTS*

8020 A. General Discussion

Quality assurance and quality control (QA/QC) are essential elements of laboratory bioassay procedures. A good QA/QC program provides framework and criteria for assessing data quality, including a well-defined chain of responsibility, explicit data quality objectives, procedures and protocols for testing, and a mechanism for identifying and correcting potential problems. Elements to be included in a quality assurance plan (QAP) are outlined in Section 1020A; other resources for developing a comprehensive QAP for laboratory toxicity testing programs are available.¹⁻⁷ As a minimum, QAPs for laboratories performing aquatic toxicity testing should provide specific guidance on data quality objectives, test procedures, sample handling, data management, internal quality control, and corrective action.

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8020 B. Elements of QA/QC

1. Data Quality Objectives

Data quality objectives (DQO) are either qualitative or quantitative statements describing the overall acceptable uncertainty in results or decisions derived from environmental data. Such objectives for evaluating toxicity must ensure that information obtained will provide an accurate and precise estimate of environmental effects. They identify the types of measurements to be made, the allowable bias, and desired precision of measurements.

Accuracy is defined as the degree of agreement between an observed value and the true value or an accepted reference value. For water quality parameters, a measurement of accuracy might include calibration against a known standard. For toxicity testing, a reference toxicant test (i.e., exposing the test organism to a contaminated matrix of known toxicity) can be used as a measurement of accuracy of organism response). Precision is defined as the degree of agreement among repeated measurements collected under identical conditions and usually is described by a measure of variance (e.g., variance, standard deviation, coefficient of variation). For toxicity testing, precision of organism response can be described in a control chart of responses to a reference toxicant.

If the response (e.g., survival) of test organisms exposed to a sediment/water sample is significantly different from the response to a reference or control, then the organism has been affected by the sample. Traditionally, decisions of statistical significance are made at $\alpha = 0.05$. This means that the probability of a false positive result (detecting a difference when in fact none exists) must remain below 5%. Data quality objectives must control levels of bias (i.e., the difference between the measured value and true value) and precision to ensure that statistical significance is not affected by measurement error.

Minimum data quality objectives should be provided for: water quality in the test chamber (e.g., temperature, salinity, alkalinity, hardness, dissolved oxygen, pH, and ammonia); frequency and acceptable limits; minimum control survival; sensitivity of test organisms (e.g., reference toxicant testing); and frequency and number of observations.

Limits for bias and desired levels of precision generally are not stipulated in standardized test protocols described herein, but should be specified in the laboratory's own manual of Standard Operating Procedures (SOPs). Performance criteria (e.g., acceptable levels for control survival or water quality measurements) for most of these categories may be found in the test protocols for the organism of interest.

2. Test Procedures

Test procedures describe how to make all routine measurements associated with toxicity testing and related QA/QC activities. Follow these procedures to ensure integrity and quality of data.

Use SOPs and standardized data forms to ensure quality and consistency of toxicological testing and reporting. Write SOPs for all routine laboratory activities and periodically review and

update them. Examples of quality control checklists, project schedule lists, procedural checklists, and test and reference toxicant procedures are available.¹⁻⁵

Steps taken in the laboratory to reduce the potential for bias include blind testing, random assignment of organisms to test chambers, statistical designs (e.g., the randomized block), procedures to prevent cross-contamination, confirmation and witnessing of recorded observations, use of reference toxicant tests, and control charting.

Blind testing, where the experimental treatment is unknown to the analyst, prevents the analyst from applying biases upon the treatments based upon any preconceived expectations.

Use randomized designs to eliminate bias due to test chamber position within the test array. The completely randomized block, in which treatments are allocated to the experimental units at random, is the simplest form of the design. Each unit has an equal chance of receiving a particular treatment. In addition, process the units in a random order at all subsequent stages of a test where order has the potential to affect results. For example, position test containers maintained in a water bath under a light source randomly within the testing area. When replicates receiving a single treatment are placed together, observed differences cannot be attributed solely to treatment; differences may have resulted from placement as well as treatment. Discussions of randomized block design, completely randomized block design, and other statistical aspects of experiment design are available.⁶⁻¹⁰

During setup and conduct of toxicity tests, prevent contamination from an external source and cross-contamination between treatments. Preventive measures include cleaning of equipment between contact with treatments, proper conditioning of laboratory test apparatus to minimize leaching, and covering test chambers to minimize loss of volatiles or extraneous contamination. Preferably, also analyze food, dilution water, and control water/sediment periodically for background contamination.

Periodic double checks of observations, data entry, and calculations and witnessing of all raw data sheets (i.e., having a coworker review and sign each raw data sheet) are good preventative steps for early identification and correction of errors. Important preventative procedures should include counting animals twice to assure accuracy before adding them to the test chamber and periodic confirmation of calibration and measurements, particularly if environmental factors seem to be out of range.

Use reference toxicant tests to assess sensitivity of test organisms. Plot results from reference toxicant tests on control charts (Section 1020B) to determine whether the sensitivity of test organisms to a given reference toxicant is within a predetermined range of acceptability. Construct control charts by plotting successive values, for example, LC50s, for a reference toxicant, and evaluating temporal changes in sensitivity. Recalculate the mean and standard deviation with each plot until the statistics stabilize. Evaluate individual values in relation to the mean and standard deviation. Procedures for developing and using control charts are described in detail in Section 1020B and elsewhere.¹¹

3. Sample Handling, Storage, and Shipment

Consistency in sample handling and tracking is most important for the testing of samples where legal ramifications are possible. To make technically sound decisions that withstand potential litigation, it is essential that samples be handled appropriately and be traceable to their source. Key components of this QA/QC element include established chain-of-custody procedures as well as procedures for sample sieving, subdividing, homogenization, compositing, storage, and monitoring.

Chain-of-custody procedures require an unbroken record of possession of a sample from its collection through analysis or testing, disposal, and possibly up to and during a court proceeding.¹² The goals of chain-of-custody are twofold: to ensure that the sample collected was the sample tested and to ensure that the sample has not been tampered with or altered in any way. Chain-of-custody can be accomplished through use of custody seals and sample tracking forms. Examples of such forms are available.^{1,12}

a. Water and wastewater: Guidance for handling effluent samples under the National Pollutant Discharge Elimination System (NPDES) program dictates that samples are to be stored at 4°C and that the lapsed time from collection to initiation of testing should not exceed 36 h.² However, holding times may be adjusted depending on study objectives and other specific logistical considerations (e.g., shipment of samples from remote areas). If water samples are to be stored, keep headspace to a minimum. Record condition of sample (e.g., pH, temperature, salinity, conductivity, dissolved oxygen) upon receipt and prior to use. Before storage, floating debris may be removed, if necessary, by pouring water samples through 2- to 4-mm mesh sieve. If there is a possibility of interference due to the presence of indigenous organisms that show predation, competition, etc., pass samples through a 60- μ m mesh sieve.² However, if volatile contaminants are of concern, take care to minimize aeration during collection, handling, storage, and testing.

b. Sediment: Sediment samples may require sieving before testing. Decisions regarding sieving are driven by presence of debris, such as twigs or leaves, that may impact recovery of test animals at test termination and/or the presence of indigenous species in the sample that may serve as food for, compete with, or actually prey upon the test organism. In any case, test results can be biased. If sieving is required, press-sieve all sediments without adding water (including reference and control sediments) before testing. In most cases, a 0.5-mm screen size is sufficient for removing predators while larger sieves may be used for removing debris. Recommendations regarding sieving of test material usually are found in specific standardized test protocols.

Depending on test objectives, samples may be composited, homogenized, and/or subdivided before testing. Use clean, non-contaminating containers and implements to handle and store samples. Suggested materials are stainless steel, TFE, Lexan®, high-density polyethylene, and glass. Other appropriate materials may be specified. Homogenize sediments to a consistent color and texture. Samples may be homogenized by hand with a spatula made of noncontaminating materials, or by mechanical mixing. Verify efficiency of homogenization by chemical analysis.

Sediments frequently are stored before testing. Current guidance for dredged material evaluations permits pre-test storage of sediment samples for up to 8 weeks from time of collection.¹³ Preferably store sediment samples at 4°C with zero headspace or under an inert gas such as argon. Rehomogenize samples just before testing.

Maximum time limits for storage of sediments prior to testing are of concern; test samples as soon after collection as possible.

4. Data Recording, Reduction, Validation, and Reporting

Quality control of recording, reducing, validating, and reporting data is necessary for production of complete and scientifically defensible reports. Issues to be considered include maintenance of laboratory notebooks, data management, reporting and validation procedures, identification and handling of unacceptable data and outliers, measurements of completeness and comparability, and procedures for data archival.

Standardization of data recording facilitates electronic transfer and manipulation of data. At a minimum, standardize procedures for intralaboratory data entry. Identify no-data entries with a mark, “—,” to indicate that data were not omitted. Use abbreviations for names of personnel and routine laboratory observations to reduce data recording and entry time; standardize these whenever possible. Attach a list of definitions and code descriptions to data sheets and project files. Record data in indelible ink; make corrections by drawing a single line through the mistake, correcting the mistake, dating and initialing the correction, and giving an initialed explanation for the lined-out data in a footnote at the bottom of the data sheet. More detailed guidance on maintaining laboratory notebooks can be found elsewhere.¹⁴

Validate all original data at each level of transcription (e.g., entering data from bound laboratory notebooks into computer databases). Arrange for an independent QA/QC review on a minimum of 10% of the data. Review laboratory records daily for outliers or unusual observations so that any necessary corrective action can be taken.

Criteria for establishing outlier values are program-specific. Toxicity endpoint outliers such as survival, growth, or reproduction may be more important than water quality outliers. Depending on program requirements, identify outliers and either accept them as “real” or reject and selectively remove. If outliers are removed from a data set, note this and clearly justify the reason. For example, an outlier for mortality in a given replicate might be reasonably excluded from a data set when it is clearly related to spurious low dissolved oxygen. If there is no rational explanation for the outlier, it must be assumed that the value is real and representative of the variability of the test system.

Completeness and comparability are measures of data quality. Completeness is a measure of the amount of data obtained versus the amount of data originally intended for collection. Generally 80 to 90% is an acceptable level of completeness for water quality data. However, endpoint data, such as survival or reproduction, should be 100% complete; otherwise the statistical power of the test may be compromised. If data are less than 80% complete, use professional judgment to assess the usefulness of the data for decision-making. Comparability is defined as the confidence with which one data set can be compared against another. Comparability and confidence can be enhanced through

interlaboratory calibration including use of reference toxicants and control charts.

5. Internal Quality Control Checks

Internal quality control checks are "in-house" procedures implemented by the laboratory to ensure high-quality data. Internal quality control checks include review of documentation to determine that all samples are tested, sample holding times are not exceeded, holding conditions are acceptable, test protocols followed, instruments calibrated and maintained, and control survival and water quality conditions are within acceptable ranges. Other important issues are verification of the taxonomy and viability of test organisms.

Document source and culture history of test organisms. If possible, preserve a subsample of the test organisms for future identification in the event of aberrant toxicity. The age, size, and/or maturity of the test organisms usually are specified in the test protocol; verify these. Specify appropriate holding time and acclimation procedures either in the test protocols or the laboratory's SOPs; ensure that resulting documentation is available for audit.

Two widely accepted ways to assess test organism viability are the use of test-validation controls and reference toxicant tests. A test validation control is a group of organisms that, with the exception of the treatment factor, are handled in a manner identical to the other organisms in the test. Acceptable levels of mortality in the test validation controls for most acute lethality tests are limited to $\leq 10\%$ (i.e., survival $\geq 90\%$). If less than 90% survival is achieved in the test validation control, the test is considered invalid and must be repeated. For chronic sublethal tests, the test validation control also may include acceptable limits for other endpoint data such as growth and reproduction. Reference toxicant tests are designed to assess sensitivity to a specific contaminant. In a reference toxicant test, organisms are exposed to a range of concentrations of a single contaminant or contaminant mixture in water-only exposures and an LC50 (usually 48 or 96 h) is calculated. Evaluate results of reference toxicant tests in a laboratory control chart (see Section 8020B.2).

Before testing, develop guidance for defining deviations, deficiencies, and appropriate corrective action. Corrective action may be required when a deficiency or deviation from planning documents or procedures is discovered or when there are deviations from established data quality objectives.

Deviations are defined as data outside the range specified in data quality objectives. Out-of-compliance data may be due to deviations in test protocols or deficiencies associated with toxicological tests. Examples of deviations from the DQO in toxicity tests include excessive control mortality, out-of-range water quality conditions, lack of randomization, lack of required reference, control, and/or out-of-range reference toxicant results.

Poor control survival, loss of control over exposure conditions, major mechanical errors, or mishandling of test organisms may result in a decision to retest. However, brief episodes of out-of-range water quality conditions or incomplete test monitoring information may require only that data be flagged and qualified. A number of typical test deviations and suggested corrective actions are summarized in Table 8020:I.

Corrective actions may include, but are not limited to, reviewing the data and calculations, identifying and qualifying suspi-

TABLE 8020:I. SUMMARY OF TYPICAL TEST DEVIATIONS AND NEED FOR RETESTING

Deviation	Need for Retesting	
	Required	Possible*
Lack of test array randomization		✓
Testing not blind		✓
Required references, controls not tested	✓	
Test chambers not identical		✓
Test containers broken or misplaced		✓
Mean control mortality exceeds acceptable limits	✓	
Excessive control mortality in a single replicate		✓
Test organisms not randomly assigned to test chambers		✓
Test organisms not from the same population		✓
Test organisms not all the same species or species complex	✓	
Test organism holding time exceeded		✓
Water quality parameters consistently out of range	✓	
Brief episodes of out-of-range water quality parameters		✓
Test monitoring documentation incomplete		✓
Sample holding times exceeded	✓†	
Sample storage conditions outside acceptable ranges		✓†

* If not retested, data may have to be qualified depending on study objectives.

† Unless evidence provided to clearly show that sample quality (physico-chemistry and contaminant levels) has not been affected.

cious data, and retesting. Review all "out-of-limit" events as soon as data are tabulated and validated.

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8030 MUTAGENESIS*

8030 A. Introduction

1. Significance

Mutagenesis may be defined as the induction of a heritable change in an organism's genetic material. Studies of human cancer have established that a mutagenic event is very likely the initiating factor in some kinds of cancers. Most known carcinogenic chemicals and radiations also are mutagenic. Therefore, demonstration of mutagenic activity suggests that the substance may be (but need not be) carcinogenic. The common association between mutagenic activity and carcinogenicity is the basis for using short-term mutagenesis tests with bacteria or cultured cells to detect potential carcinogens.

It is also known that mutagens of natural origin are ubiquitous in the environment.¹ Therefore, the relationship between mutagenic activity of an environmental sample and chemical pollutants in the sample must be examined very carefully in order to draw conclusions about the source of mutagenic activity.

2. Selection of Method

Many tests to detect mutagenicity exist.² Tests using bacteria, particularly the *Salmonella* microsomal mutagenicity (Ames) test, are most common.^{3,4} The latter test uses nonvirulent "tester strains" of *Salmonella typhimurium*. Because many mutagenic chemicals require some metabolic processing by enzymes that are lacking in the bacteria, mammalian enzyme preparations can be added. The test is simple, inexpensive, and sensitive, gives results in 2 d, and shows good correlation between mutagenicity/nonmutagenicity and carcinogenicity/noncarcinogenicity in rodents. Disadvantages of the test are that some mutagens active in mammalian cells will not be detected, carcinogens that are not also mutagens (e.g., asbestos) will not be detected, and the relative potency of mutagens in the test does not necessarily correlate with the carcinogenic potency in mammals. Although

the test provides *qualitative* information regarding mutagens, it shows great variability among laboratories in *quantitative* terms. However, it is of great value as a preliminary screening test.

Because environmental samples are complex mixtures, no standard application of the *Salmonella* microsomal mutagenicity test is possible.^{5,6} The design of an individual test will depend on the sample and circumstances and the desired information regarding mutagenic activity. For example, to determine whether a particular mutagen is present, a specific chemical extraction procedure may be necessary. If a concentration step is used, it must be considered in reporting results. To determine whether a particular treatment process (e.g., chlorination or ozonation) leads to mutagen production, samples should be assayed before and after treatment. Sample preparation depends on the nature of materials in the raw water.^{6,7} The methods described here are for the Ames plate incorporation test. Generally they are applicable to samples soluble in aqueous or organic solvents. Within limits, they will provide preliminary data on the presence of mutagenic materials. For definitive information, tailor the Ames test and sample preparation to the specific situation. When reporting results, state the sample preparation used.

3. Sample Collection, Storage, and Preparation

Because of variability among samples, a single procedure for sample preparation cannot be provided, but general principles apply:⁵⁻⁹

Make tests as soon as possible after sample collection. Delay in testing will be accompanied by a progressive loss in mutagenic activity, no matter how the sample is stored. To minimize loss of mutagenic activity, store samples at or below -20°C , under an inert atmosphere (N_2 , argon), and protected from light. Many mutagens, particularly polycyclic aromatic hydrocarbons (PAHs) are readily photooxidized to nonmutagenic, but still toxic, compounds. Add no preservatives.

Exposure of the *Salmonella* tester strains requires the sample to be in a solvent compatible with the aqueous suspension of the bacteria. Dimethylsulfoxide (DMSO) is used frequently. If the

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Joint Task Group: Yi Y. Wang (chair), Jack W. Anderson

sample is in a volatile solvent such as dichloromethane (DCM) or hexane, add DMSO and remove the organic solvent with a stream of N₂, leaving behind the relatively less volatile DMSO. This is called solvent-exchanging. After the sample has been transferred to DMSO analyze immediately.¹⁰

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8030 B. *Salmonella* Microsomal Mutagenicity Test

1. General Discussion

a. Principle: Tester strains of *S. typhimurium* require the amino acid histidine for growth. Reversion of the tester strains from histidine dependence to independence, i.e., reversion of the histidine requirement, is evidence of mutagenicity. The bacteria can be cultivated on simple media and show reproducible responses to test mutagens. The bacteria are exposed to the sample, with or without additional activating enzymes, and are plated on minimal agar containing a trace amount of histidine. Mutants (i.e., revertants to histidine independence) are able to grow and form macroscopic colonies. The dose-response can be quantified by varying sample concentration and counting revertant colonies per plate at each concentration. The number of revertants per unit dose of sample is calculated with statistical methods.

b. Tester strains: The tester strains currently most widely used in testing environmental samples are TA98 and TA100. Strains TA97a and TA102 can be used but are found to give high and variable rates of background mutation. Because the different strains are reverted by different classes of mutagens, using multiple strains provides information on the nature of the mutagenic chemical(s) present. These strains also contain the R-factor plasmid pKM101, which confers resistance to ampicillin. Other commonly used strains include TA1535 and TA1538, which do not contain the R-factor plasmid. Details of the tester strain mutations and other available strains have been published.¹⁻⁴ Quality assurance requirements and procedures,^{5,6} as well as data production and analysis methods,^{7,8} are available.

Tester strains presently are available without cost by written request to Dr. Bruce N. Ames, Department of Biochemistry, University of California, Berkeley, California 94720.

A number of related tester strains have been developed and validated for detecting specific classes of mutagenic compounds. Tester strains TA98NR (nitroreductase) and TA98/1,8-DNP₆ (1,8-dinitropyrene) have been used widely to detect the presence of nitro-substituted compounds. Tester strain TA98NR is deficient in "classical" nitroreductase and has lower response to 2-nitrofluorene and 1-nitropyrene, for example, than the activity detected in tester strain TA98. Tester strain TA98/1,8 DNP₆ is deficient in acetyl-CoA (acetylcholinesterase): *N*-hydroxy-arylamine *O*-acetyltransferase, an enzyme important for the metabolic activity of compounds such as 2-nitrofluorene and 1,8-dinitropyrene.

For detecting aromatic amines and nitro-PAHs, investigators^{9,10} have developed *Salmonella* tester strains (derived from the standard tester strains) that have elevated levels of enzymes important for the metabolic activation of nitro-substituted compounds. For example, tester strains YG1021 and YG1024 have elevated levels of the enzyme nitroreductase and OAT (*O*-acetyltransferase) activity.^{10,11} These strains are derived from tester strains TA98. There are also tester strains YG 1026 and YG 1029 that are derived from tester strain TA100, but have elevated nitroreductase and OAT enzymes, respectively. Tester strains carrying both nitroreductase and OAT have also been developed and are sensitive to nitro-aromatic compounds tested.¹² The YG tester strains have been used in investigations for toxic compounds in water and the samples were more mutagenic

with YG1024 than TA98.¹³ The protocol for growing YG strains incorporates overnight incubation with antibiotics.

The investigators who developed the YG tester strains have incorporated adding antibiotics to the overnight broth culture. They also add these to TA98 and TA100. For tester strains YG1021, YG1026, YG1024, and YG1029, ampicillin (25 µg/mL) and tetracycline (12.5 µg/mL) were added. For tester strains YG1041 and YG1042, ampicillin (25 µg/mL) and kanamycin (25 µg/mL) were added. These antibiotics are used to test for the strain characterization as well.

2. Apparatus

- Autoclave*: See Section 9030B.3.
- Water bath*, reciprocating, for use at 37°C.
- Water bath* for use at 45°C to 50°C.
- Incubator* for use at 37°C.
- Refrigerator or cold room*.
- Freezer*, -80°C (or liquid nitrogen refrigerator).
- pH meter*.
- Centrifuge*, capable of 10 000 × g.
- Vortex mixer*.
- Magnetic stir-plate and stirring bars*.
- Hot plate*.
- Colony counter*, manual and automatic (optional).
- Microscope*, dissecting or light.
- Micropipettor*, 10 µL, 100 µL, 500 µL, and 5 mL.

3. Media and Reagents

a. Nutrient broth: Use dehydrated nutrient broth prepared in accordance with manufacturer's directions.*

b. Nutrient agar plates: These are used to test for tester strain characteristics including crystal violet sensitivity. Add 15 g agar/L nutrient broth, before sterilizing.† Mix well and autoclave at 121°C for 15 min with slow exhaust. Remove from autoclave and let cool to about 50°C. Pour 25 to 30 mL into 100-mm petri plates and let harden on a level surface. To evaporate excess moisture, hold covered plates in a clean, draft-free environment overnight. Store prepared plates in a tightly covered container in the refrigerator.

c. Vogel-Bonner medium E (50X): This is an inorganic salt medium² used in the preparation of minimal agar. To prepare 1 L of 50X concentrate, heat 670 mL water to 45°C and add in order (making sure each salt is completely dissolved before adding the next) 10 g magnesium sulfate heptahydrate, MgSO₄·7H₂O; 100 g citric acid monohydrate; 500 g potassium phosphate, dibasic K₂HPO₄ (anhydrous); and 175 g sodium ammonium phosphate, NaNH₄HPO₄·4H₂O. Make up to 1 L with water in a loosely capped 2-L flask or bottle and sterilize by autoclaving for 20 min at 121°C. After cooling, tighten cap and store at room temperature.

d. Glucose solution, 40%: To 600 mL water add 400 g D-glucose. Stir and make up to 1 L with water. Mix well and sterilize in a loosely capped flask by autoclaving for 20 min at

121°C (slow exhaust). Alternatively, dispense to 250-mL or 500-mL bottles with rubber-lined screw caps before autoclaving. Leave caps loose during autoclaving. Tighten after solutions have cooled to room temperature.

e. Minimal agar plates for use in the mutagenicity test. To 930 mL water in a 2-L flask add 15 g agar and a magnetic stirring bar. Mix, cap loosely, and autoclave for 20 min at 121°C, with slow exhaust. Remove from autoclave, cool slightly, and add the following sterile solutions slowly with continuous stirring: 20 mL 50X Vogel-Bonner medium E and 50 mL 40% glucose. Mixing is facilitated if the salts and glucose solutions are first warmed to 45°C.

Place agar in a 45°C water bath or dry temperature block and pour approximately 25 to 30 mL into 15-mm × 100-mm petri plates. Let agar harden on a level surface and cool to room temperature. To let excess moisture evaporate, hold plates covered in a clean, draft-free area overnight or up to 2 d. A convenient method of plate storage is to return them to the plastic bags in which they were originally packaged and to seal the bags securely with tape. Long-term storage at room temperature is acceptable. If plates are stored under refrigeration let them come to room temperature before use. Autoclave and discard any plates showing contamination.

f. Histidine-biotin solution, 0.5 mM: This solution is added to the top agar in the proportion of 10 mL to 100 mL top agar. (It provides a necessary trace of histidine to permit bacteria to undergo a few cell divisions. The tester strains also are biotin-dependent, but as this requirement is the result of a gene deletion, it cannot be reverted.) Add 12.4 mg D-biotin to 100 mL water. Dissolve by heating to the boiling point and add 9.6 mg L-histidine-HCl. Sterilize by filtering through a 0.22-µm-pore-diam filter or by autoclaving for 20 min at 121°C, with slow exhaust.

g. Top agar:

Agar	6 g
Sodium chloride, NaCl	5 g
Distilled water	1 L

Add magnetic stirring bar, mix and autoclave for 20 min at 121°C, with slow exhaust. While agar is still melted, mix thoroughly and dispense 100-mL portions into sterile screw-capped bottles of a convenient size (100 to 250 mL). Alternatively, make top agar in 100-mL amounts (0.6 g agar, 0.5 g NaCl, 100 mL H₂O) and autoclave in loosely capped bottles. Cool to room temperature, tighten caps, and store at 4°C. Before use remelt top agar in a boiling water bath or microwave oven and add 10 mL sterile 0.5 mM histidine-biotin solution. Hold top agar in a 45°C water bath or dry heat device.

*h. Phosphate-buffered saline (PBS)*¹⁴ for washing bacteria in the micro suspension mutagenicity test method.¹⁵ To 900 mL distilled water add 8.0 g sodium chloride, NaCl; 0.2 g potassium chloride, KCl; 0.2 g potassium phosphate, monobasic, KH₂PO₄; 0.1 g magnesium chloride hexahydrate, MgCl₂·6H₂O; and 1.15 g sodium phosphate, dibasic, Na₂HPO₄.

Dissolve completely and add 0.10 g calcium chloride, CaCl₂, dissolved in a little water. Adjust to pH 7.4 with either HCl or NaOH as appropriate. Make up to 1 L. Sterilize by filtration through a 0.22-µm-pore-diam filter or equivalent.

* Oxoid #2, Oxoid Ltd., Basingstoke, Hants, England, available in U.S.A. from KC Biological, Inc., Lenexa, KS, or equivalent.

† Bacteriological agar, BBL Select, Oxoid #L28, or equivalent.

i. *Sodium phosphate buffer*, pH 7.4, used in S9 mix (see ¶ 1*m* below). Prepare stock solutions:

1) *Sodium phosphate*, monobasic, monohydrate, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 13.8 g/500 mL water.

2) *Sodium phosphate*, dibasic, anhydrous, Na_2HPO_4 , 14.2 g/500 mL water.

Mix 60 mL Solution 1) with 440 mL Solution 2). Check pH and adjust if necessary to pH 7.4 by adding more of one of the stock solutions. To lower pH add Solution 1); to raise it add Solution 2). Sterilize by autoclaving for 20 min at 121°C with slow exhaust.

j. *Ampicillin stock solution*, 8 mg/mL: Make a solution of 0.80 g ampicillin trihydrate in 100 mL 0.02*N* NaOH. Sterilize by filtering through a 0.22- μm -pore-diam membrane filter. Store in capped glass bottle at 4°C. Add 3.15 mL/L master plate agar solution and pour plates as usual.

k. *Master plate agar*: To a 2-L flask add 15 g agar, 914 mL water, and a magnetic stirring bar. Mix and autoclave for 20 min at 121°C with slow exhaust. Remove from autoclave and add, with stirring, 20 mL 50X Vogel-Bonner medium E salts; 50 mL 40% glucose; 10 mL sterile L-histidine-HCl solution (0.5 g/100 mL); 10 mL sterile D-biotin solution (12.2 mg/100 mL); and 3.15 mL ampicillin stock solution for ampicillin-fortified plates. Eliminate ampicillin if growing tester strain, such as TA153, that does not have the pKM101 plasmid. (NOTE: The final concentration of histidine in the master plates is approximately fivefold greater than in top agar. Clearly label plates to distinguish them from minimal agar plates. For strains without the R-factor plasmid, omit ampicillin.)

l. *Crystal violet solution*, 0.1%: Dissolve 0.1 g in 100 mL water. Mix well and store at 4°C in screw-cap glass bottle in the dark. Use to confirm presence of the *rfa* mutation.

m. *S9 mix*: S9, a cell-free fraction prepared by homogenization and centrifugation of rat liver (or other tissue) at $9000 \times g$ for 10 min, is added when metabolic activation is required. Prepare S9 from the liver of rats pretreated with polychlorinated biphenyls (PCBs, Aroclor 1254) to increase activity of liver enzymes.² Unless animal facilities are available, preferably obtain S9 commercially.‡

Because S9 contains temperature-sensitive enzymes, store frozen at -80°C or below and thaw only for immediate use. Each mutagen may have an optimum concentration of S9 for maximal mutagenic activity. Because this optimum cannot be specified in advance, and the amount of sample often is limited, standardize on an S9 concentration. Between 20 and 40 mg protein/mL S9 is common, but consistency is essential.

Standardize S9 according to protein content. Determine protein content of a small portion of undiluted S9.¹⁶ Freshly prepared S9 has a protein content of about 40 mg/mL.² Adjust protein concentration immediately before use to the desired concentration with 0.1*M* sodium phosphate buffer, pH 7.2-7.4, or with 0.15*M* KCl. Check for sterility by spreading 0.1 mL on a minimal agar plate containing histidine and biotin and incubate

for 2 d at 37°C. Discard contaminated S9 (more than 10 colonies/0.1 mL).

In an assay add cofactors to provide necessary reducing activity for the cytochrome enzymes. Add reduced nicotinamide adenine dinucleotide phosphate (NADPH) or a NADPH generating system consisting of nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), and MgCl_2 . The combination of S9 and cofactors is termed "S9 mix." Ideally, select the amount of S9 in the mix to give optimum mutagenic response with the sample. Practically, use either 4 mL or 10 mL S9/100 mL S9 mix (i.e., 4% or 10% S9, equivalent to approximately 1.6 mg or 4.0 mg protein/mL S9 mix, respectively).² For consistency adjust the protein concentration in S9 mix to these concentrations. Once prepared, keep S9 mix on ice and use immediately; do not refreeze.

Use the following stock solutions for preparing S9 mix:

1) *Potassium chloride*, KCl, 1.65*M*: Dissolve 12.3 g KCl in 80 mL water and make up to 100 mL. Autoclave for 20 min at 121°C and store at room temperature.

2) *Magnesium chloride hexahydrate*, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.4*M*: Dissolve 8.13 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in 80 mL distilled water and make up to 100 mL. Autoclave for 20 min at 121°C and store at room temperature.

3) *Sodium phosphate buffer*: See ¶ 3*i* above.

4) *NADP (nicotine adenine dinucleotide phosphate)*, 0.1*M*: Dissolve 743 mg in 80 mL sterile water and make up to 100 mL with sterile water. Sterilize by filtering through a 0.22- μm -pore-diam membrane filter. Store 5- or 10-mL portions at -20°C for up to 6 months. (NOTE: The weight given here is the formula weight of anhydrous free acid. The sodium salt typically is used and it may have associated water. Thus the amount required will vary from lot to lot. Most suppliers provide a specification sheet with the calculated formula weight. Use this value in preparing the solution. Typical values range from 750 to 825.)

5) *Glucose-6-phosphate*, 1*M*: Dissolve 6.5 g in 20 mL sterile water and make up to 25 mL. Sterilize by filtering through a 0.22- μm -pore-diam membrane filter. Store 5- or 10-mL portions at -20°C for up to 6 months.

To prepare 50 mL S9 mix, add in order, to a vessel in an ice bath: 18.75 mL or 15.75 mL sterile water; 25 mL 0.2*M* sodium phosphate buffer; 2.0 mL 0.1*M* NADP; 0.25 mL 1.0*M* glucose-6-phosphate; 1.0 mL 1.65*M* KCl; 1.0 mL 0.4*M* $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; and 2.0 or 5.0 mL rat liver S9. (NOTE: The amount of S9 can be varied to obtain desired protein concentration. Preferably use either 2 mL (4%) or 5 mL (10%) of rat liver S9/50 mL S9 mix.² Adjust water volume to maintain concentrations of other reagents.)

Prepare S9 mix immediately before use. Discard any unused portion. Do not refreeze.

4. Tester Strain Stock Cultures

a. *Preparation of stock cultures*: Tester bacteria are available (see ¶ 1*b*) as small paper disks saturated with the *Salmonella* culture, sealed in small sterile plastic bags with a little agar. On receipt, aseptically remove disks and make subcultures by wiping the disk across an agar master plate and then placing disk in sterile nutrient broth. Adjust volume of nutrient broth, depending on how many frozen stock vials are to be produced. To a culture grown overnight in nutrient broth add dimethylsulfoxide (DMSO

‡ AMC Cancer Research Center and Hospital, c/o Dr. Elias Balbinder, 6401 W. Colfax Ave., Lakewood, CO 80214; Litron Laboratories, 1351 Mt. Hope Ave., Suite 207, Rochester NY 14620; Microbiological Associates, c/o Dr. Steve Haworth, 5221 River Road, Bethesda, MD 20816; MolTox, 335 Paint Branch Drive, College Park, MD, 20742; or Organon Teknika, 1 Technology Court, Malvern, PA 19355.

spectrophotometric grade, 0.09 mL DMSO/mL culture). Mix well and dispense aseptically into sterile 1.5-mL cryotubes, filling each tube almost to the top. Label, freeze in crushed dry ice, and store at -80°C or in liquid nitrogen refrigerator. If frozen cultures are to be used repeatedly do not allow them to thaw, because this tends to increase the spontaneous background mutation rate and the chance of contamination. If freezer facilities are not available, less preferably maintain strains by repeated subculture and confirm strain characteristics at each mutagenicity test.² To prepare bacteria for a test remove a small amount of the frozen culture with a sterile spatula and inoculate a broth culture. Cultures also can be preserved by lyophilization.²

As an alternative to repeatedly sampling the frozen stocks use the "master plate" method.² Streak a drop of a broth culture or a small piece of the frozen culture on an ampicillin-master agar plate for tester strains with the pKM101 plasmid, or streak on a His/Bio-master agar plate (His/Bio only) for strains without plasmids. Minimal agar plates also can be used if subsequently fortified with histidine, biotin, and, if required, ampicillin.¹⁷ The plate may be stored in a refrigerator for up to 2 weeks. Use well-separated colonies from this plate to initiate broth cultures. Prepare new master plates from broth cultures initiated from the frozen stocks.

b. Characterization of tester strains: Characterize tester strains immediately on receipt and preferably as a part of each mutagenesis assay. The procedures described herein are for strains TA98 and TA100. Procedures for other strains are available.²⁻⁴ Strains TA98 and TA100 require histidine and biotin for growth. In addition, they contain the *rfa* mutation affecting the cell wall, the *uvrB* deletion leading to reduced DNA repair capacity, and the R-factor plasmid pKM101 conferring resistance to ampicillin.

Confirm the histidine requirement by streaking a sample of broth culture on a minimal agar plate containing biotin, but lacking histidine. The biotin requirement is the result of a deletion and cannot revert. No bacterial growth should be seen on the histidine-deficient plate.

The *rfa* mutation renders the cell wall permeable to large molecules; growth of the tester strains is inhibited by crystal violet. To test for this inhibition, add 0.1 mL of broth culture to 2 mL melted top agar, and spread the mixture on a nutrient agar plate. Place a sterile 6.4-mm-($\frac{1}{4}$ -in.-)diam disk of filter paper[§] in center of plate and add 10 μL 0.1% crystal violet solution to the filter paper. The crystal violet will diffuse into the agar; sensitive strains show a clear zone around the paper disk, indicating growth inhibition. The *uvrB* deletion confers increased sensitivity to ultraviolet light, which can be demonstrated by comparison with the wild-type strain.¹⁷ The presence of the R-factor plasmid allows tester strains to grow on agar containing ampicillin.

The strains are reverted by different mechanisms and their response to chemical mutagens depends on the mode of interaction of the chemical with the bacterial DNA. Strain TA98 is reverted by frame-shift mutagens that generally are large molecules such as polycyclic aromatic hydrocarbons. The reading frame of the DNA is shifted by the deletion or addition of a base pair. Strain TA100 is reverted by mutagens that cause base-pair

TABLE 8030:I. DIAGNOSTIC MUTAGENS FOR TESTER STRAINS
TA98 AND TA100

Chemical	Amount per Plate	S9	Histidine Revertants per Plate		Animal Toxicity
			TA98	TA100	
Sodium azide	1.5 μg	—	3.0	3000	Highly toxic
Daunomycin	6.0 μg	—	3123	47	Toxic
Methyl methanesulfonate	1.0 μL	—	23	2730	Carcinogen
2-Aminofluorene	10 μg	+	6194	3026	Carcinogen
Benzo(a)pyrene	1.0 μg	+	143	937	Carcinogen

substitutions in the bacterial DNA. In this mutation, an adenine-thymine base pair is replaced by a guanine-cytosine pair, or vice-versa. For strains TA98 and TA100 diagnostic mutagens are recommended;² these are relatively more mutagenic for one strain than for the other. See Table 8030:I. In using diagnostic mutagens, relative mutagenic activity is important; absolute activity varies between laboratories. When a test is conducted with the diagnostic mutagens, a dose-response curve is generated in the same manner as with samples.

c. Safety precautions: The tester strains were derived from *S. typhimurium* type LT2, which is of low virulence. They have been modified further to increase their sensitivity to mutagens but incidentally to decrease their virulence. Aside from deliberate ingestion, the tester strains do not present a health hazard to workers. Nevertheless, good laboratory practice dictates certain precautions. Never pipet by mouth. Do not eat, drink, or smoke in the laboratory. Autoclave all live cultures and culture plates with colonies before disposal.

Handle plates or cultures or materials containing positive control mutagens or carcinogens as hazardous materials. Treat all test plates (except for negative controls) and other materials prepared for testing as potentially hazardous, and dispose of them accordingly.

5. Procedure

a. Without metabolic activation: Conduct all operations in subdued light or under dim incandescent, nonactinic lighting. Yellow "bug-lights" or "gold" fluorescent tubes that emit very little ultraviolet radiation are satisfactory.

1) Culture preparation—Prepare overnight broth cultures of tester strains by inoculation with either frozen stock or master plate colonies. Each test plate requires 0.1 mL of broth culture (approximately 1 to 2×10^8 bacteria). Prepare the volume of broth culture accordingly. Incubate cultures overnight (10 to 12 h) at 37°C , with shaking at approximately 210 rpm to ensure adequate aeration.

2) Test material preparation—Most mutagens are soluble in the recommended DMSO. Other relatively nontoxic, nonmutagenic solvents such as ethanol or acetone may be used provided that excessive toxicity is absent.¹⁸ Because many compounds are water-soluble, use water whenever possible. When using a volatile solvent take precautions to prevent solvent evaporation before it is added to the top agar or incubation mixture. Keep solutions with volatile solvents tightly capped and on ice. The final concentration of solvent in the top agar should not exceed

§ Schleicher & Schuell No. 740-E or equivalent.

5%, except when the solvent is water. Dissolve test material at the highest concentration to be tested and prepare appropriate dilutions. Use a concentration range of at least three logs, with test concentrations at half-log intervals, e.g., 1, 3, 10, 30, 100, 300, and 1000 $\mu\text{g}/\text{plate}$. It may be more convenient to specify dose levels for water samples in terms of original water volume equivalents per plate. In the latter case, mutagenic activity, if present, has been detected in the range of 0.05 to 2.0 liter-equivalents.¹⁹ If negative results are obtained at the highest concentration and no toxicity to the bacteria is apparent, increase concentration of test material to 5 or even 10 mg/plate,^{5,7} unless limited by toxicity or precipitation. Prepare sample solutions immediately before use; do not store.

3) Plate incorporation test—Prepare at least three plates for each concentration of test material with minimal base agar. Remove melted top agar tubes from water bath or dry temperature block (43 to 45°C) and add to 2 mL of melted top agar: 0.1 mL bacteria, 0.1 mL or less (usually 0.05 mL) test compound or solvent control, and either 0.5 mL S9 mix for with-S9 testing, or 0.5 mL phosphate buffer for minus-S9 testing. It may be convenient to place the required number of tubes containing melted top agar in the 45°C water bath in advance and to add bacteria and test material just before pouring test plates. Briefly mix with a vortex mixer, pour on minimal agar plates, and immediately tilt and rotate plates so that the top agar forms a uniform layer before hardening. Place plates on a level surface and let top agar solidify. (Use a carpenter's level to confirm that the surface is level.) Work quickly. Negative controls receive solvent (e.g., DMSO) or no addition to the mixture of bacteria and top agar. When the top agar has solidified, invert and incubate plates in the dark at 37°C.

4) Scoring plates—Count revertant (histidine-independent) colonies after 48 to 72 h. Longer incubation will result in a very slight increase in the number of revertant colonies but also will increase the chance of contamination. Hold incubation time constant in any test series. Because *Salmonella* strains are motile, the size of colonies depends on whether their bacteria were at the surface or embedded in the top agar. Surface colonies tend to be large (up to 2 mm), circular, and flat while embedded colonies are small (<1 mm) and dense. All colonies are a uniform light cream color, slightly translucent, with a smooth surface and a regular smooth edge to the surface colonies. The appearance of colonies with differing morphology indicates contamination; discard plate and check stock culture for purity. Colony size is inversely proportional to the number of revertants per plate because of exhaustion of nutrients. A trace of histidine in the top agar enables the bacteria to undergo several replications before the histidine is exhausted; it effectively increases the number of bacteria at risk for mutagenesis. This replication also serves as an indicator of toxicity. Replication of nonrevertant bacteria gives a hazy appearance to the top agar between the revertant colonies. If this background lawn replication is absent, it indicates excessive toxicity to the bacteria, and an unreliable mutation test. The variability of the numbers of revertant colonies between replicate plates is usually about 10 to 15%. It tends to become less with increased experience of the analyst.

Plates with bacteria only show a characteristic number of spontaneous revertant colonies. Among a group of eight laboratories the spontaneous revertant range for strain TA98 was 15 to 75 and for strain TA100 between 60 and 220 colonies/plate.⁷

Within a given laboratory, these values should remain relatively constant. If marked variation occurs, investigate immediately by recharacterizing the strains and confirming media formulation. These numbers may be slightly different on plates with S9 mix. Plates that have received bacteria exposed to the solvent only may show slightly higher numbers of spontaneous revertants.

b. With metabolic activation:

1) Without preincubation—Except for the addition of S9 mix to the standard plate test to convert certain mutagenic chemicals to their active forms, the general procedure is the same as that described above.

Add 0.5 mL S9 mix to the top agar, test material, and bacteria as described above. Incubate and score plates as above. Include a positive control mutagen when using S9 to demonstrate that the preparation is active and to detect possible variation between batches of S9. Recommended control compounds requiring metabolic activation include 2-aminofluorene,² 7,12-dimethylbenz(a)-anthracene, and 2-acetylaminofluorene.⁷

2) With preincubation—To enhance test sensitivity for materials or mixtures containing low levels of mutagenic activity, preincubate the bacteria with the test material, with or without S9 mix.²⁰ To a 13- × 100-mm tube containing the test material in 0.1 mL sodium phosphate buffer add 0.5 mL S9 mix and 0.1 mL of desired tester strain (approximately 1 to 2 × 10⁸ cells). If S9 mix is not used, replace it with 0.1 mL buffer. Incubate at room temperature or at 37°C for 20 min. Add 2 mL melted top agar and pour mixture on a minimal agar base plate as above. The temperature and length of preincubation affect the yield of revertant colonies and should be optimized and standardized for the material being tested. The S9 mix remains metabolically active for 30 to 45 min; avoid prolonged incubation.

3) Microsuspension modification—When the amount of material available for testing is small or the mutagenic activity is very weak, the microsuspension assay may be used.²¹ Prepare overnight bacterial cultures as described above. Harvest the bacteria by centrifugation (5000 × g, 10 min, 4°C) and resuspend pellet in a volume that is one-tenth the original volume, using phosphate buffered saline (PBS) or sodium phosphate buffer. Add the following ingredients, in order, to a 12- × 75-mm sterile glass tube: 0.1 mL bacterial suspension, 0.1 mL S9 mix or PBS, and 0.005 mL test compound dissolved in a compatible solvent. After incubation for up to 90 min, add top agar, mix, and pour plates. Sensitivity is increased up to tenfold or more because the bacteria are in contact with higher concentrations of the test material or mutagenic metabolites during preincubation. However, the toxic effects of the material also may be increased. Make standard assay to provide a reference. Optimize incubation conditions for the particular material being tested.

6. Data Presentation and Analysis

a. Data presentation: Preferably report raw data (i.e., revertant colonies per plate, including control plates) as well as interpreted data.^{7,8} If this is not feasible, indicate means of replicate plates, number of replicates, number of experiments, and a measure of the variability (e.g., standard deviation). In cases with a clearly positive result, present data graphically to facilitate comparisons. Direct comparisons of different samples, such as extracts from different waters, usually are done in a single experiment that

subsequently is replicated. Presentation of data transformed to revertants per weight of test material or water volume equivalents or to ratios of treated to control cultures is acceptable only if the original data also are available. Report any pretreatment used on the sample.

b. Data analysis: Usually it will be apparent if the test material is mutagenic, so involved statistical analysis of the results is not required. However, when dealing with weakly mutagenic materials, preferably establish objective criteria for deciding whether a material is mutagenic or contains mutagenic substances. An important first criterion is that a reproducible dose-response relationship can be demonstrated, i.e., the number of revertants per plate is proportional to the amount of test material added per plate over some part of the range of amounts tested. As a guide, use the *modified twofold increase rule*,^{1,5} which states that a test is considered positive if two consecutive dose levels, or the highest nontoxic dose level, produced a response at least twice that of the solvent control, and at least two of these consecutive doses showed a dose-response relationship. If results are in doubt, further testing is indicated, perhaps with test modifications.

It may be desirable, for quantitative comparison purposes, to express mutagenic activity in terms of *Salmonella* revertants per microgram of material or per water volume equivalent, but often the dose-response curve is nonlinear. With low amounts of test material, the number of revertants per plate may increase with increasing amount of material but beyond a certain amount, the number of revertants per plate no longer increases, and even may decline. The reasons for this nonlinearity include toxicity of the test material to the bacteria and limited solubility of the test material in the aqueous medium. Therefore, mutagenic activity of an unknown compound or mixture often is described in terms of the slope of the linear portion of the dose-response curve with low amounts of test material. Statistical methods have been described for deciding which points on these nonlinear curves to use in defining specific mutagenic activity of the sample.^{14,22}

Failure to demonstrate mutagenic activity with one or more *Salmonella* tester strains does not amount to proof that the sample contains no mutagenic material. The individual strains have certain mutagens to which they are sensitive, the concentration of S9 may be critical, and some mutagens and carcinogens are not mutagenic for any commonly used strain. Therefore, qualify conclusions regarding mutagenic activity on the basis of the characteristics of the tester strains used as well as the test conditions.^{2-4,8}

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8050 BACTERIAL BIOLUMINESCENCE*

8050 A. Introduction

The bacterial bioluminescence test (BBT) is a metabolic inhibition test that uses a standardized suspension of luminescent bacteria as test organisms under standardized conditions. This test method provides a rapid, reliable, and convenient means of determining acute toxicity. The BBT has been validated for a variety of environmental applications including effluent monitoring, groundwater testing, sediment testing, hazardous wastes testing, assessing the efficiency of bioremediation processes, and general biomonitoring.

Luminescent bacteria possess several attributes that support their use for toxicity testing. Their small size (less than 1 μm in diameter) provides a very high surface-to-volume ratio. This feature, as well as the relatively simple morphology and lack of membrane-sided compartmentalization of internal functions, provides many target sites at or near the cytoplasmic membrane.

* Approved by Standard Methods Committee, 2001.
Joint Task Group: Devon A. Cancilla (chair).

In bacteria, many metabolic pathways that function in respiration, oxidative phosphorylation, osmotic stabilization, and transport of chemicals and protons into and out of the cell are located within, or very near, the cytoplasmic membrane. The luciferase pathway, which functions as a shunt for electrons directly to oxygen at the level of reduced flavin mononucleotide, also is located within the cell membrane complex.

These cellular characterizations, coupled with the fact that bacterial respiration is 10 to 100 times greater than that of mammalian cells, provide for a dynamic metabolic system that can be quantitated easily and accurately by measuring the rate of light output from a bacterial suspension. Typically, the suspension contains approximately 10^6 individual organisms. The light intensity is substantial (well within the operating range of common light sensors), and the number of organisms compensates for variations among individuals that may influence results of tests on statistically limited numbers of individuals.

8050 B. Bacterial Bioluminescence Test

1. General Discussion

Principle: Certain strains of luminescent bacteria divert up to 10% of their respiratory energy into a specific metabolic pathway that converts chemical energy into visible light. This pathway is intrinsically tied to respiration; any change in cellular respiration or disruption of cell structures results in a change in respiration with a concurrent change in the rate of bioluminescence.

In the BBT, living bioluminescent bacteria, *Vibrio fischeri* (formerly *Photobacterium phosphoreum*), are exposed to a test sample and the toxic effect of the sample on the bacteria is measured. The test organism (*Vibrio fischeri*) and testing equipment are available commercially.^{1,2} Data reported in the literature were produced in tests using standard, commercially available, freeze-dried strains of the bacterium, allowing meaningful comparison of results of tests from different laboratories.³⁻⁵ The test system measures the light output of rehydrated lyophilized bacteria after exposure to a specified dilution series of a sample, and compares it to the light output of a control blank (i.e., bacterial cell suspension in diluent only). It is assumed that the difference in light production is due to the effect of the sample. The degree of light loss (degree of metabolic inhibition in the bacteria) indicates the degree of sample toxicity. A dose-response curve is determined, on which the IC₅₀ is located. The IC₅₀ is the inhibiting concentration of a sample causing a 50% decrease in the bacteria light output under defined conditions of exposure time and test temperature. Positive controls of either

standard phenol or ZnSO₄ solutions are used to assess ongoing method performance.

2. Apparatus

- Photometer* and computer with appropriate software.†
- Glass cuvettes, 12 × 50 mm, disposable, with flat bottoms.
- Pipettor,‡ adjustable (200 to 1000 μL).
- Pipettor§ with disposable 0.5-mL syringe tips.
- Laboratory glassware, including sample bottles, 30- to 250-mL beakers, and volumetric flasks.
- Spatulas, stainless steel.
- Weighboats, plastic.
- Water bottles.

3. Reagents and Materials

- Bacteria (*Vibrio fischeri*), freeze-dried, in reagent vials.
- Sodium chloride, NaCl, solid, reagent-grade.
- Diluent: Make up a 2 % NaCl solution with deionized/distilled water. Use to dilute sample and reagent as necessary.
- Sodium thiosulfate, Na₂S₂O₃, reagent-grade.

* Microtox ® Model 500 Analyzer, available from Azur Environmental, Carlsbad, CA, or equivalent.

† Microtox Data and Reduction System, or equivalent.

‡ Eppendorf or equivalent.

§ Eppendorf Repeater ® or equivalent.

- e. *Sodium thiosulfate stock solution*: Make up a 1% $\text{Na}_2\text{S}_2\text{O}_3$ solution with deionized/distilled water.
- f. *Water*, ultrapure, for use as reconstitution solution.
- g. *Phenol*, reagent-grade, crystalline.
- h. *Zinc sulfate*, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, reagent-grade.
- i. *Plastic film*.||
- j. *Disposable wipes*.#

4. Procedure

a. *Sample collection, handling, and treatment*: Preferably collect 250 to 500 mL sample in nontoxic jars or bottles leaving no airspace. Test sample as soon as possible, preferably 24 to 48 h after collecting it, but if necessary, store it in the dark at 4°C. Prompt testing avoids possible changes in sample toxicity with time.

Centrifuge sample if significant turbidity is present. Transfer cleared sample to a new container. (NOTE: Material causing turbidity also may have toxic effects.) Other tests are available (solid-phase testing procedures⁶) if turbid samples must be analyzed as received.

The test bacteria are adversely affected by pH conditions below 6.0 and above 8.0. Adjust sample pH with either NaOH or HCl as needed. If over-titration occurs, discard sample and start over.

Osmotically adjust samples to a final concentration of 2.0% as NaCl. Pour 50 g homogenized sample into a 100-mL beaker. Tare out the beaker plus sample and add 1.00 g solid AR grade NaCl crystals to the beaker. Stir with a disposable glass pipet until all crystals are completely dissolved.

The test organism is sensitive to chlorine. If necessary, dechlorinate samples using a 10 000-mg/L stock solution of sodium thiosulfate. Prepare sodium thiosulfate stock using the diluent described in ¶ 3c. Prepare the sample by adding sufficient sodium thiosulfate stock solution to obtain a final sodium thiosulfate concentration of 100 mg/L. For example, addition of 100 μL 10 000-mg/L sodium thiosulfate stock to 10 mL sample will give a sample containing 100 mg/L sodium thiosulfate.

b. *Positive control preparation*: Include positive controls of either phenol or ZnSO_4 as part of the basic test protocol. Depending on the number of samples to be tested, analyze positive controls at least once a day. Phenol and ZnSO_4 ordinarily exhibit IC_{50} values of 13 to 26 mg/L and 5 to 12 mg/L, respectively.** Prepare a 100-mg/L stock solution of either phenol or ZnSO_4 by carefully weighing and transferring 100 mg of the specific positive control into a clean and rinsed (with dilution water) 1000-mL volumetric flask. Protect the phenol standard from light and prepare in an amber-colored or aluminum-foil-wrapped volumetric flask. Although both standards will last for 3 to 4 months, preferably prepare standards on each day of testing.

c. *Analyzer and bacteria (*Vibrio fischeri*) preparation*: Power up the analyzer at least 30 min before analyses are to be performed to allow the unit to bring the reagent well, incubator wells, and read well to their pre-set temperatures. The green "ready light" indicates that the system is ready.

Place clean, unused cuvettes in the reagent well (maintained at $5.5^\circ\text{C} \pm 1^\circ\text{C}$), and in the sample wells of the incubator block

Key: ♦ = reagent well, ○ = empty well

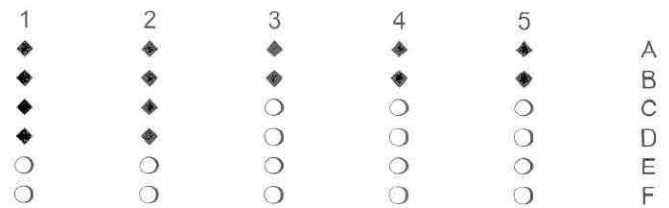


Figure 8050:1. Incubator diagram for acute toxicity testing of one sample at multiple dilutions.

(maintained at $15^\circ\text{C} \pm 0.5^\circ\text{C}$) as required for the number of samples to be analyzed. To set up analyzer for acute toxicity testing of one sample at multiple dilutions, follow the incubator diagram of Figure 8050:1.

Place 1000 μL reconstitution solution (ultrapure water) into the cuvette within the reagent well. Allow 10 min for solution to come to temperature. Take a reagent vial from the freezer, tap the pellet to bottom of vial, and remove seal.

Take cuvette containing distilled water from reagent well, and place lip of cuvette on top of reagent vial. Quickly pour the distilled water into reagent vial (slow reconstitution causes lysing of the bacteria and low reagent light levels). Swirl reagent vial three or four times only (overmixing will warm the reagent), pour reagent mixture back into cuvette, and place cuvette back in reagent well.

Mix reconstituted reagent with the 500- μL pipettor by filling and dispensing 20 times for uniform dispersal of the reagent. Allow 20 min for reconstituted bacterial reagent to stabilize.

d. *Sample preparation*: Pipet 500 μL diluent (2% NaCl solution) into each of the cuvettes in Wells B1, B2, B3, B4, B5, D1, and D2. Pipet 1000 μL diluent into each cuvette in Wells A1, A2, A3, A4, A5, and C1. The cuvette in Well C2 receives no diluent. Pipet 1000 μL osmotically adjusted sample into cuvette in Well C2. Pipet additional 1000 μL of osmotically adjusted sample into the cuvette in Well C1 (diluent already present) and mix using pipettor.

To prepare the series of 1:2 dilutions, transfer 1000 μL from C1 to A5. Mix using pipettor. Transfer 1000 μL from A5 to A4 and mix using pipettor. Transfer 1000 μL from A4 to A3 and mix using pipettor. Transfer 1000 μL from A3 to A2 and mix using pipettor. Discard 1000 μL from cuvette A2. The cuvette in A1 contains only diluent and is the control blank (negative control). Wait 5 min for temperature equilibration.

The test cuvette sample concentrations (%) now are:

1	2	3	4	5	Test volumes are:
0.00	3.125	6.25	12.5	25.0	A Each cuvette in Row A contains 1000 μL .
0.00	0.00	0.00	0.00	0.00	B Each cuvette in Row B contains 500 μL .
50.0	100.0				C Each cuvette in Row C contains 1000 μL .
0.00	0.00				D Each cuvette in Row D contains 500 μL .

|| Parafilm ® or equivalent.

Kimwipes ® or equivalent.

** Reagent Certificates of Performance, which report IC_{50} values for phenol and ZnSO_4 , are available from Azur Environmental for each lot number of bacteria.

e. *Bacterial reagent addition:* Using the repeat pipettor, add 10 μL reconstituted reagent from the reagent well (about one million bacteria) into each cuvette in Wells B1, B2, B3, B4, B5, D1, and D2. These cuvettes already contain 500 μL diluent. When dispensing reagent into cuvettes, do not remove reagent cuvette or sample cuvettes from the incubator while adding reagent.

After addition of 10 μL reagent to each of the cuvettes, mix each reagent dilution by gently removing each cuvette and gently tapping the bottom of each two to three times. Wait 15 min after reagent dilution for reagent stabilization. During this period, set up the recording software as required.

f. *Measurement of light emission:* Measure the time-zero levels of light from the cuvettes of diluted reagent in Rows B and D before adding sample dilutions from the cuvettes directly above them in Rows A and C, respectively.

After the 15-min reagent-stabilization period, calibrate analyzer by obtaining a reading for Cuvette B1 (nontoxic reagent control), according to instrument manufacturer's instructions. Later measurements read during the test are expressed as values on a comparative scale, based on actual performance of reagent currently in use. Read the time-zero light level in each cuvette, and return cuvette promptly to its incubation well. The sequence of cuvette reading is from the control cuvette up the concentration gradient (B1 to D2). After all of the time-zero readings have been acquired, the software automatically starts a timer to track the addition of reagent with sample. Perform the following steps immediately.

g. *Sample-to-reagent transfer:* Using a pipettor adjusted to deliver 500 μL , transfer 500 μL from cuvette in Well A1 to cuvette in Well B1. Gently mix by drawing up the mixture with the pipettor. Repeat transfers for A2 to B2, A3 to B3, A4 to B4, A5 to B5, C1 to D1, and C2 to D2.

Immediately after the last transfer, allow the computer to note the elapsed time since the last time-zero light production (I_0) reading. This is the time spent making the sample transfers, and is used by the system to set the timing of the I_t ($t = 5$ or 15 min) reading intervals to ensure that each cuvette receives equal exposure time before its I_t reading.

At this point in the test protocol, the test cuvette sample concentrations (%) are:

1	2	3	4	5	Test volumes are:
0.00	3.125	6.25	12.5	25.0	A Each cuvette in Row A contains 500 μL .
0.00	1.563	3.125	6.25	12.5	B Each cuvette in Row B contains 1010 μL .
50.0	100.0				C Each cuvette in Row C contains 500 μL .
25.0	50.0				D Each cuvette in Row D contains 1010 μL .

When computer timer sounds, read I_5 light levels as prompted. The pauses between readings correspond to elapsed time during sample transfer. When computer timer sounds again, read I_{15} light levels as prompted. End test according to directions on computer screen.

5. Data Reduction, Reporting, and Interpretation

The system estimates an IC50 value and the 95% confidence range of this value. A narrow confidence range means more

accurate results. Information presented at the end of the testing period includes: the dilution (as a percentage) at which each measurement was taken, the time-zero light readings (I_0), the light readings at both 5 and 15 min (I_t), and gamma values corrected by the control ratio ($CR = \text{control } I_t \div \text{control } I_0$), which corrects for natural light loss over time in a blank cuvette (diluent and reagent only). The gamma (Γ) value is then the ratio of light lost to light remaining after exposure of the reagent to the sample. It is calculated by the equation:

$$\Gamma_t = [(CR \times I_0) \div I_t] - 1$$

If an IC50 value can be calculated from the data, it is displayed in the units specified when the test parameters were outlined. By definition, IC50 = gamma 1. Not every concentration necessarily produces a valid gamma. The progression of test values should show a dose-response relationship, meaning that gamma values should increase with increasing sample concentration (unless there is no inhibiting effect at any concentration and all values are similar). At least three data points are used to make final calculations. If the light output for the entire range of dilutions is zero, re-test sample with lower dilutions. Also, if all dilutions resulted in no significant light decrease, flag all data points with an asterisk.

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8070 P450 REPORTER GENE RESPONSE TO DIOXIN-LIKE ORGANIC COMPOUNDS*

8070 A. Introduction

1. General Discussion

A cell culture of human liver cancer cells can be used to detect the presence of toxic and/or carcinogenic organic compounds in environmental samples. A sample of water, soil, aquatic sediment, or tissue is extracted with a solvent to remove semivolatile organic compounds, and a small amount of the extract is applied to a culture well containing cells attached to the bottom of the well in medium. After exposure, the cells are rinsed and lysed, the cell fragments are removed by centrifugation, and the extract is tested for luminescence. A luminescent enzyme (luciferase) is produced by the cells if dioxin-like compounds were present in the extract, because a reporter gene (plasmid) from the firefly has been attached to the human chromosome at the site induced by dioxin and other planar compounds (at the CYP1A1 gene). The amount of light produced, which is quantified by a luminometer, is a function of the concentrations and induction potency of the organic compounds in the extract. Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD) has the strongest affinity for the re-

SWEET, L.I., D.F. TRAVERS & P.G. MEIER. 1997. Chronic toxicity evaluation of wastewater treatment plant effluents with bioluminescent bacteria: A comparison with invertebrates and fish. *Environ. Toxicol. Chem.* 16: 2187.

ceptor on the cell membrane (Ah-receptor), and therefore will be detected at the lowest concentration.

This assay is both a detection system and a meaningful biological response to the toxicants in environmental samples. It can be used to screen environmental samples for the presence of some of the most toxic and carcinogenic compounds. Only those compounds that are dioxin-like and that attach to the Ah-receptor will induce the CYP1A1 gene and result in the production of luciferase. Such induction would occur in humans or wildlife, including aquatic species, if these compounds came in contact with their tissues. Induction of the CYP1A1 gene is one of the key factors used in designating a compound a carcinogen. High levels of such induction in fish (P450 measurements) have been shown to correlate with histological damage and reduced reproductive capacity.

Another procedure based on these principles is available.¹

2. Reference

1. AMERICAN SOCIETY FOR TESTING AND MATERIALS. 2004. Guide for measuring the presence of planar organic compounds which induce CYP1A, using reporter gene test systems. E1853 M-98, Annual Book of ASTM Standards, Vol. 11.05. American Soc. Testing & Materials, W. Conshohocken, Pa.

* Approved by Standard Methods Committee, 2001.
Joint Task Group: Jack W. Anderson (chair).

8070 B. The P450 RGS Test

1. Principle and Significance

The P450 Reporter Gene System (RGS) is a biomarker test for the detection of toxic and/or carcinogenic organic compounds, using a transgenic cell line (101L)* derived from the human hepatoma cell line (HepG2). Under appropriate test conditions, induction of the CYP1A1 gene in mammalian cells normally results in the production of the enzyme P450IA1. This response is evidence that the cells have been exposed to one or more xenobiotic organic compounds, including dioxins, furans, copla-

nar polychlorinated biphenyls (PCBs), and several polycyclic aromatic hydrocarbons (PAHs). Detection of induction has been made rapid and inexpensive by the stable integration of a firefly plasmid, such that Ah-receptor binding and subsequent transcription results in the production of the luminescent enzyme, luciferase. This RGS test has shown concentration-response relationships using dilutions of TCDD, 2,3,7,8-TCDF, five coplanar PCBs, and eight PAHs, and has responded to application of extracts from environmental samples.¹⁻⁴ Environmental samples (water, tissue, soil, or aquatic sediments) may be extracted by established methods, and the extracts applied to the test system to detect the presence of toxic and carcinogenic organic compounds.

* Cells are available from Columbia Analytical Services, Vista, CA 92083.

The organic compounds that induce CYP1A1 site on the chromosome are toxic, often carcinogenic, and several have been shown to bioconcentrate and biomagnify. Various birds, mammals, and fish exposed to these compounds have exhibited physiological, reproductive, and histopathological effects.⁶⁻⁷ Millions of dollars are spent annually on chemical analyses of water, food, wildlife, soil, and aquatic sediment because of concern for contamination from these compounds. Use of a screening tool such as the RGS will permit selection of a subset for further chemical characterization.

2. Test Summary

Details of the culture and testing methods have been published.⁸⁻¹⁰ For most environmental samples, dichloromethane (DCM) is used as the extraction solvent, but for extraction of ash samples for dioxins/furans, toluene is used. Initial dry weight (determined on a separate subsample) of extracted sample, final volume of solvent containing the extracted material (0.5 or 1.0 mL), and amount applied to the cells are recorded. Regardless of the extraction solvent or system, DMSO, iso-octane, or another low-toxicity solvent is the final solvent applied to the cells. Extracts of environmental samples are added to individual wells (six-well plates) containing approximately one million cells that have been growing for 3 d. Usual exposure time is 16 h. To examine the relationship between chlorinated hydrocarbons and PAHs in an extract, response is measured at 6 h and 16 h, because PAHs reach peak induction at 6 h and PCBs and dioxins reach peak induction at 16 h.¹¹ If information is required on only the chlorinated inducers (dioxins, furans, coplanar PCBs) first clean the extracts of PAHs with silica gel columns. Application of 20 μ L solvent produces a low background (control) induction when applied to cells in 2 mL of culture medium. The luminescence (in relative light units, RLU) of the combined cytoplasm from the cells in each well is compared to that of other replicate wells, the solvent control, and reference toxicants (TCDD, and five concentrations of a dioxin/furan mixture) using a 96-well luminometer. The mean RLU of the control wells is set to unity. Mean RLUs of samples are first recorded as fold induction (times background), which is derived by dividing by the mean RLU of the solvent (background control). A dioxin/furan standard curve [toxic equivalents (TEQ) vs. fold induction] is prepared from the results of each test, and the slope is used to adjust fold induction values to TEQs (in ng/g dry weight). The RGS response represents the integrated CYP1A1 induction from all dioxin-like compounds present in the extract. Because standard curves have been prepared for the response to benzo(a)pyrene

and a mixture of dioxins and furans, the results of the assay may be expressed as micrograms of benzo(a)pyrene equivalents, or nanograms of TCDD TEC per gram dry weight or per liter.

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8071 COMET/SINGLE-CELL GEL ELECTROPHORESIS ASSAY FOR DETECTION OF DNA DAMAGE*

8071 A. Introduction

1. Significance

Many pathological conditions are initiated by an increased incidence of DNA damage. The most common example is carcinogenesis initiated by mutagens resulting from DNA damage. Changes in the normal function of an organism resulting from contaminant exposure begin at the cellular and molecular levels.¹⁻³ DNA damage may be manifest in the form of base alterations, adduct formation, strand breaks, and cross-linkages.⁴ Of these, the most prevalent type of genetic damage is the DNA single-strand break; many waterborne contaminants have been shown to cause dose-dependent incidences of strand breaks.⁴⁻¹¹ Strand breaks may be introduced directly by genotoxic compounds, through the induction of apoptosis or necrosis, secondarily through the interaction with oxygen radicals or other reactive intermediates, or as a consequence of excision repair enzymes.¹²⁻¹⁴ In addition to a linkage with cancer, studies have demonstrated that increases in cellular DNA damage precede or correspond with reduced growth, abnormal development, and reduced survival of adults, embryos, and larvae.¹⁵⁻¹⁷

The comet assay is a simple, sensitive, and versatile method for the detection of DNA damage in individual cells. The assay can be applied to cells collected from virtually any eukaryotic organism, and can be used to detect DNA damage in vitro, in vivo, and in situ resulting from exposure to a broad spectrum of environmental contaminants.

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* Approved by Standard Methods Committee, 2001.

Joint Task Group: Scott A. Steinert (chair), Marsha C. Black, Richard F. Lee, P.J.E. Quintana, Raymond R. Tice.

8071 B. Comet/Single-Cell Gel Electrophoresis Assay

1. Principle and Application

Under alkaline conditions the comet assay facilitates the detection of DNA single-strand breaks and alkaline labile sites in individual cells, and can determine their abundance relative to control or reference cells.¹⁻³ A small number of cells, as few as 5000 or so, are immobilized in an agarose gel by suspending the cells in liquid agarose, which is then placed on a microscope slide and allowed to solidify at low temperature. The cells are lysed in a buffer containing detergents and high concentrations of salt; the immobilized DNA is then denatured under alkaline conditions and subjected to electrophoresis. DNA strand breaks cause localized relaxation and fragmentation of the tightly coiled DNA molecule. During electrophoresis the relaxed and broken strands of negatively charged DNA migrate away from the nucleus toward the anode. When stained with a fluorescent DNA stain and viewed through an epifluorescence microscope, each nucleus and associated tail of damaged strands of DNA resembles a comet. Cells with increased DNA damage resulting from strand breaks have a greater fraction of the total DNA migrating away from the immobilized nuclear DNA; cells with increased damage resulting from crosslinks exhibit reduced DNA migration. The migration of DNA away from the nucleus (i.e., comet tail length) can be measured by eye with an ocular micrometer. Alternatively, the comets can be classified into different categories associated with increased migration based on appearance. Comet tail length, percentage of migrated DNA, tail moment (tail length multiplied by fraction of DNA in the tail), and other DNA migration values can be calculated with the use of image analysis software.

Electrophoresis under alkaline conditions (pH>13) allows for the detection of single-strand breaks and alkaline-labile lesions, whereas neutral pH conditions facilitate the detection of double-strand breaks.⁴ Various sample treatments can be used to identify specific types of DNA damage or to preserve damage at sites of DNA repair.⁵ Nuclease digestion steps can be used to introduce strand breaks at sites of specific lesions. With this approach, oxidative base damage can be detected by the use of endonuclease III or formamido pyrimidine glycosylase,⁶ as well as DNA modifications resulting from exposure to ultraviolet (UV) light through the use of T4 endonuclease V.⁷ The variation used depends on the type of cell being examined, the types of DNA damage the researcher wishes to detect, and the imaging/analysis capabilities of the laboratory conducting the assay.

For the analysis of DNA damage, this assay offers a number of advantages; damage to the DNA in individual cells is measured, only very small numbers of cells need to be sampled, the assay can be performed on practically any eukaryotic cell type, and it has been shown in comparative studies to be very sensitive.^{8,9}

Significantly elevated levels of DNA damage detected by this method have been reported in cells collected from organisms in polluted sites compared to reference sites.¹⁰⁻¹² These studies have shown that increases in cellular DNA damage correspond to decreased growth, survival, and development, and correlate with significant increases in contaminant body burdens or highly elevated metabolic demands.

Because the method is simple and cost-effective, it has been used to screen the genotoxicity of various compounds on cells *in vitro*,¹³ as well as to evaluate the dose-dependent antioxidant (protective) properties of various compounds.^{3,5,7,14,15}

2. Apparatus

- a. *Water bath(s)*, set at temperatures of 35 to 40°C and 70°C.
- b. *Centrifuge*, capable of exerting a 600-g force, handling 1.5-mL microcentrifuge tubes.
- c. *Microwave oven*, small, low-energy model.
- d. *Microscope slides*, 7.6 cm × 2.5 cm × 1 mm, clear glass with frosted label area.
- e. *Electrophoresis chamber*, submarine. Larger chambers with platforms approximately 20 × 20 cm allow more gels to be processed at one time.
- f. *Electrophoresis power supply*, generally capable of delivering DC constant current of 300 mA and a voltage gradient of 0.4 to 1.3 V/cm.
- g. *Epifluorescence microscope*, with excitation and emission filters appropriate for the fluorescent stain being used (e.g., ethidium bromide, 510- to 560-nm excitation filter and 590-nm barrier emission filter).
- h. *Ocular micrometer*, for measuring DNA migration distance by eye if an image system is not available.
- i. *Image analysis system* (optional), comprising a CCD camera attached to the microscope and connected to a computer loaded with the appropriate image-analysis software. Commercial systems are available specifically for comet assay applications.

3. Reagents and Materials

- a. *Slide coating agarose*, electrophoresis-grade, for slide base coat dissolved in TAE working buffer, to a final concentration of 1.0%. Store melted and solidified agarose in sealed glassware at room temperature, for up to several months if necessary. Before use, re-melt in a 70°C water bath or a microwave oven.
- b. *TAE buffer stock solution*, 50×: Combine 242 g Tris [tris(hydroxymethyl) aminomethane] base, 57.1 mL glacial acetic acid, 100 mL 0.5M EDTA, pH 8.0, and enough distilled water to produce a final volume of 1 L. Store in refrigerator.
- c. *TAE working buffer solution*, 1×: Dilute 10 mL stock TAE in 490 mL distilled water. Make as needed.
- d. *Cell suspension agarose (CSA)*, with low melting point (~30°C), dissolved to 0.5 to 1.0% in buffer solution (¶s e and f below) suitable for cells being studied. Solidified agarose can be stored in sealed vials at room temperature for months and re-melted in a 70°C water bath or microwave oven, then transferred to a 35 to 40°C water bath before cell preparation.
- e. *Kenny's salt solution* (for marine invertebrate cells):^{10,11,15} To 900 mL distilled water, add 23.5 g NaCl, 0.7 g KCl, 0.1 g K₂HPO₄, and 0.2 g NaHCO₃. Adjust pH to 7.5 with NaOH, bring to final volume of 1 L, and filter through a 0.22-μm filter (filter sterilize). Store at 4°C (usable for several weeks).
- f. *Phosphate-buffered saline (PBS) stock solution* (for many vertebrate cell applications):^{3,14} 10×: To 900 mL distilled water, add 1.361 g KH₂PO₄, 14.2 g Na₂HPO₄, 80.1 g NaCl, and 20.1 g

KCl. Adjust to pH 7.0, bring to final volume of 1 L, filter-sterilize, and store at 4°C.

g. *PBS working solution*: Dilute PBS stock solution, using 10 mL 10× PBS and 90 mL distilled water. After dilution pH should be ~7.4.

h. *Stock lysis solution*: Combine 146.4 g NaCl, 37.2 g EDTA, and 1.2 g Tris-HCl, in 500 mL distilled water; while stirring slowly, add 8 to 12 g NaOH to facilitate dissolution of EDTA and bring the pH to 10. Adjust volume to 1 L, filter-sterilize, and store at 4°C.

i. *Working lysis solution*: Combine 89 mL lysis stock solution, 10 mL DMSO, and 1.0 mL polyethylene glycol *p*-isooctylphenyl ether.* Bring to final volume of 100 mL. Make fresh on day of use; refrigerate at 4°C before use.

j. *Electrophoresis/unwinding solution* (pH > 13): To 968 mL distilled water add 30 mL 10N NaOH and 2 mL 0.5M EDTA (pH 8.0). Bring final volume to 1 L. Make fresh on day of use.

k. *Tris neutralization buffer*, 0.4M, pH 7.5: Mix 200 mL 1M Tris (pH 7.5) with 300 mL distilled water, for 500 mL final volume.

l. *Tris buffer*, 1M, pH 7.5: Dissolve 121.1 g Tris base in 800 mL distilled water, add 63 mL conc HCl, adjust pH to 7.5, and bring to final volume of 1 L.

m. *Ethidium bromide stock solution*: CAUTION: Use extreme care when handling; ethidium bromide is a powerful mutagen. Wear gloves and other appropriate safety protection. Dispose of contaminated items and waste properly. Dissolve 10 mg ethidium bromide (EtBr) in 1 mL distilled water, and store in light-protected container at 4°C. The solution is stable for months.

n. *Ethidium bromide working solution*: Add 10 µL EtBr stock solution to 5 mL distilled water. Store in light-protected container at room temperature; this solution is stable for weeks.

o. *Ethanol*, reagent-grade, 95%: Store in freezer at -20°C; this reagent is stable for months.

p. *Cell preparation*, suspension of selected cells in 50 to 1000 µL maintenance medium. Use maintenance medium most likely to reduce stress on cell types used.

4. Procedure

a. *Slide preparation*: NOTE: Wear gloves whenever handling slides and slidecovers; skin oils reduce adherence of agarose to the microscope slides. Prepare 100 mL of melted slide coating agarose. Solid agarose preparations can be liquefied in a microwave oven with alternating short pulses of microwaves followed by gentle swirling. Grasp each slide (¶ 2d) at label area, dip into melted agarose, and leave immersed for at least 10 s. Remove from agarose and wipe excess agarose off back of slide, and then place on a level surface and let dry for 2 h at room temperature or for 30 min in a drying oven at 37°C. Store dry slides in a moisture-free slide storage box at room temperature. Slides prepared in this manner may be stored almost indefinitely and require no further preparation before sample application.

b. *Sample preparation*: Place cells suspended in maintenance medium (¶ 3p) in a 1.5-mL centrifuge tube and centrifuge (e.g., at 600 g for 2 min) to form pellet. Carefully draw off supernatant

and discard. In most cases a cell pellet about 1 to 2 mm in diameter is more than sufficient to yield cell densities of 5 to 20 cells per microscopic field of view at 200×, when resuspended in 50 to 400 µL cell suspension agarose. Experience in estimating and adjusting cell densities will reduce the number of overlapping and superimposed nuclei encountered during scoring. Melt CSA (¶ 3d) and place in a 35 to 40°C water bath; let temperature stabilize. Gently resuspend pellet in appropriate volume of CSA at 35 to 40°C, as determined by the researcher. Before the agarose solidifies, transfer a portion of the cell suspension (e.g., 50 to 75 µL) to an agarose-coated slide. Spread sample uniformly over slide by placing a clean glass coverslip on top of the still-liquefied sample. Place slide on a level, ice-cold metal or glass surface until agarose has solidified. Then add a top coat of 50 to 75 µL CSA by slipping coverslip off slide, applying agarose, and replacing coverslip on top. Let agarose solidify as before, remove coverslip, and place slide in a glass slide jar filled with ice-cold working lysis solution (¶ 3i), so that the solution completely immerses slide area covered by sample. Minimum lysis period is usually 1 h, with no apparent maximum. However, gels can become more fragile with extended lysis times.

c. *Unwinding and electrophoresis conditions*: After lysis, rinse slides in distilled water or neutralizing buffer to remove lysis salts and detergents by immersing slides for 2 min and replacing with fresh distilled water/neutralizing buffer three times. Place slides in electrophoresis chamber and fill chamber with electrophoresis/unwinding solution to a depth of 3 to 4 mm above the slides. Set slides side by side and in contact, if necessary forming multiple rows on the chamber platform. Leave slides undisturbed in electrophoresis/unwinding solution for 15 to 60 min. (Optimum unwinding and electrophoresis times can be determined by comparing the extent of migration in untreated control target cells and target cells exposed to a DNA damaging agent (e.g., gamma radiation, hydrogen peroxide, methylmethane sulphonate). In order for historical data to be useful, the negative control cells should exhibit some level of DNA migration.) Set power supply to run for 5 to 60 min at 300 mA constant current, with a voltage gradient that may range from 0.4 to 1.3 V/cm. After electrophoresis, turn power off, remove slides from chamber, and neutralize alkali by three rinses of 2 min duration in neutralization buffer (¶ 3k). Fix DNA in the agarose gel by soaking for 5 min in ice-cold 95% ethanol. Dry slides at room temperature or in a 37°C oven. The dried slides can be stored indefinitely in slide boxes until stained and scored.

d. *Staining*: CAUTION: Wear gloves throughout these procedures, and whenever the slides are handled subsequently. Place 10 to 40 µL EtBr† working stain solution (¶ 3n) on each slide, place a coverslip on top, and remove excess stain with an absorbent tissue.

e. *Scoring*: Examine stained slides with epifluorescence microscope, optimally between 200 and 400 × magnification. (Optimal magnification depends on the size of the cells being assessed.) Ethidium bromide bound to double-stranded DNA has fluorescence excitation and emission maxima of 518 nm and 605 nm, respectively. Use excitation and barrier filters specific for

† Many fluorescent DNA stains are commercially available. While EtBr is referred to in this procedure, propidium iodide and YOYO also have been used successfully.^{4,14,16}

* Or equivalent nonionic surfactant.

this fluorescent dye. Slides viewed in this way reveal stained comets as bright fluorescent orange balls 10 to 40 μm in diameter. Conduct scoring with either an ocular micrometer (§ 2h) or an image analysis system (§ 2i). With an image analysis system, it is possible to measure the fluorescence intensity and distribution of DNA throughout the comet.¹⁴ In this way, the percentage of DNA in the comet tail, the length of the tail, tail moment (which is the product of the fraction of DNA in the tail and tail length), and numerous other measures can be determined.

Regardless of scoring method, always determine the number of comets per slide. Generally, score 50 to 100 cells per sample.^{17,18} Fewer cells would eliminate the ability to identify subpopulations of cells with altered migration among a larger population of cells with migration patterns not different from those in the control sample. Because of inherent variability within and across electrophoresis runs, preferably score two slides per sample, with 50% of the data obtained from each replicate slide. Score comets in different sectors of each slide. Avoid scoring comets near edge of slide and do not score slides with high background levels of staining. Once the field of view is randomly moved to a sector, use a systematic method of scoring, such as scoring comets from left to right starting in the upper left-hand corner until a predetermined number of comets has been counted. Do not count overlapping and superimposed comets. Ensure that slide scoring is "blind" (i.e., that the sample identity of the slide is not known during scoring).

After scoring, remove coverslips of scored slides, dry the slides, and store as permanent records that can be re-stained and viewed at a later date.

5. Data Analysis

a. Interpretation of data: Depending on the method of scoring, different types of data may be gathered including, but not limited to, ocular measurement of comet image or tail lengths, the fraction of cells with different patterns of migration, or image-analysis-based comet tail lengths, percentage of migrated DNA, tail moment, or other measurements. The distribution of migration patterns can be expressed graphically in histograms by plotting frequency of comets (Y axis) and corresponding DNA damage measurement gathered for those comets (X axis). In addition, dose-response plots can be constructed showing DNA damage (mean and standard deviation of data, Y axis) and test compound concentration (X axis).

b. Acceptability of data: Compare DNA damage levels in controls and (if study design allows) damage resulting from reference toxicants to previously gathered data to determine acceptability. If studying cells from organisms or cell lines on which it is possible to perform laboratory exposure tests, design experiments to incorporate positive controls and construct control charts to evaluate test performance (see Section 1020B.12). Prepare control chart for each combination of reference toxicant and test organism, and include the most current data in each control chart. End points from five tests are adequate for establishing a chart. Use the control charts to evaluate the cumulative trend of results from a series of samples. Recalculate mean and upper and lower control limits (± 2 SD) with each successive result.

If the value from a given test with the reference toxicant falls more than 2 SD outside the expected range, the sensitivity of the

organisms and the overall credibility of the test system may be suspect. In this case, examine test procedure for defects and repeat with a different batch of test organisms.

c. Statistics: Establish statistical methods to be used and data requirements for those methods during initial experiment design. The unit of exposure for in-vitro studies is the culture, while for in-vivo studies it is the animal. Thus, multiple cultures or multiple animals are needed per dose group to provide data for an appropriate statistical analysis. Examine the homogeneity of variance between treatments to determine whether parametric or nonparametric analysis is appropriate. Transformation of nonhomogeneous data also can be explored. If homogeneity is not achieved using transformed data, use nonparametric procedures. Linear regression analysis can be used to establish dose-response relationships, while pairwise comparisons of each treatment group against the concurrent control can be conducted. Use two-tailed statistical test if both an increase and decrease in DNA are being tested; a one-tailed test if only one is being tested.

d. Evaluation and interpretation of results: If a positive comet assay response is obtained, assess the possibility that the increase in migration is not associated with genotoxicity. Information on the extent of cytotoxicity associated with each positive dose group, the nature of the dose-response curve, the intercellular distribution of comet response at each dose, and the presence or absence of necrotic or apoptotic cells in the treated cell population may be useful. Common determinations of cytotoxicity rely on simple dye exclusion on vital staining assays. In the event that a negative comet assay response is obtained, assess the validity of the assay and the dose selection procedure. Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgment about the activity of the test substance. Reproducibility in independent experiments is considered the strongest evidence for a positive or negative call. However, results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

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8080 SEDIMENT POREWATER TESTING*

8080 A. Introduction

1. Applications

The standard approach for assessing the quality or potential toxicity of marine or estuarine sediments has been to expose macrobenthic organisms directly to whole sediments for a specified time, after which the survival of the test species was determined. Whole-sediment methods^{1,2} have limitations, including use of adult macrobenthic organisms and the use of mortality as the primary end point. In addition, the standard amphipod test protocol^{1,2} underestimates the potential toxicity of contaminated sediments because the pore water is flushed out and replaced with fresh overlying water before the start of the exposure.

The porewater toxicity test approach offers several advantages over the standard whole-sediment method. Sensitive life stages of sensitive species can be used in tests utilizing sublethal end points. There are no artifacts produced by sediment texture. A dilution series test design can be used easily for better differentiation among highly toxic samples. Whereas whole sediment preferably should be tested within 2 weeks of collection, studies indicate that pore water can be stored in the frozen state for extended periods of time without any change in toxicity.³ Most

of the studies on porewater testing have focused on marine and estuarine species.

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*Approved by Standard Methods Committee, 1997.
Joint Task Group: 20th Edition—Robert S. Carr (chair), Donald J. Reish.

8080 B. Sediment Collection and Storage

Sediment collection methods vary considerably with specific study objectives. Remove as much overlying water as possible from the sample before placing it in the sample container or adding it to a composite sample. Nearly fill sample container to minimize head space, allowing some room for sample to be

rehomogenized in its original container. Extract pore water as soon as possible after sample collection. If the sediment sample cannot be processed immediately, store on ice or refrigerate at 4°C. The toxicity of pore water extracted from refrigerated sediments can change considerably after a period of weeks or even days.

8080 C. Extraction of Sediment Pore Water

Methods that have been used for obtaining sediment pore (interstitial) water including centrifugation,¹⁻³ pressurized (pneumatic or mechanical "squeezing") extraction,⁴⁻⁸ vacuum (suction) methods,^{9,10} and equilibration methods using dialysis

membranes or fritted glass samplers.^{4,11,12} Studies comparing recovery efficiencies of trace metals and organics for different extraction methods indicate that substantial losses of nonpolar contaminants (e.g., fluoranthene and *p,p'*-DDE) can occur with

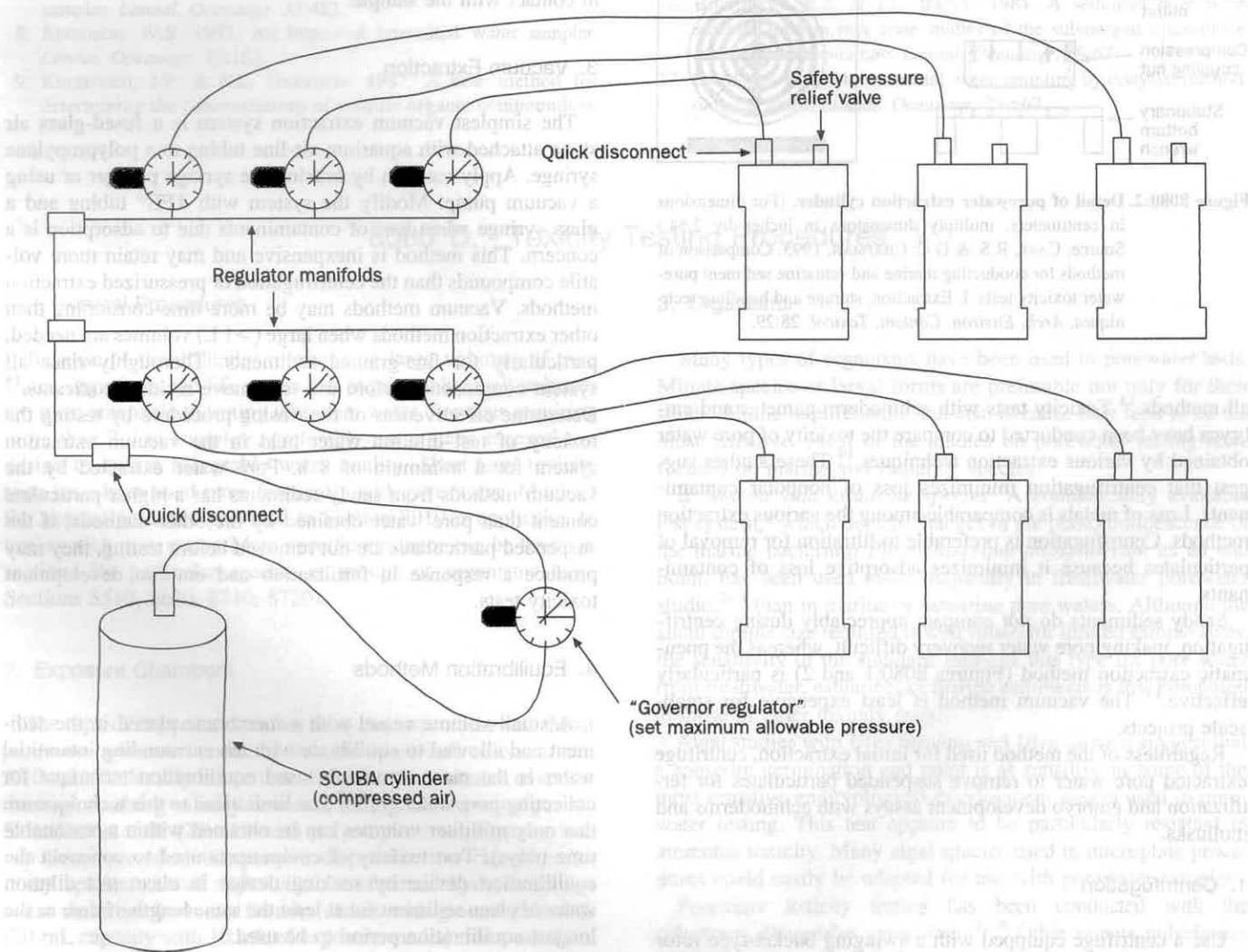


Figure 8080:1. Pneumatic system for porewater extraction. Source: CARR, R.S. & D.C. CHAPMAN. 1995. Comparison of methods for conducting marine and estuarine sediment porewater toxicity tests. I. Extraction, storage and handling techniques. *Arch. Environ. Contam. Toxicol.* 28:29.

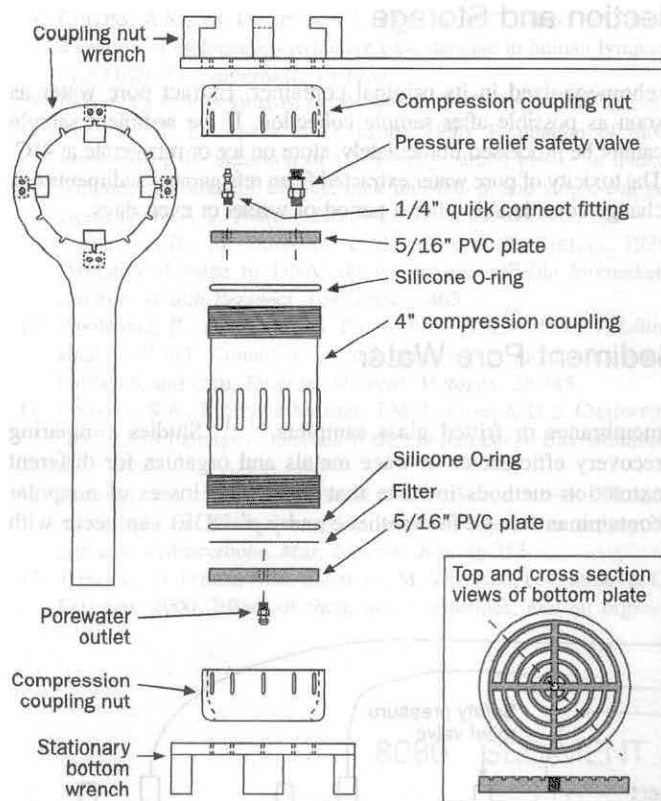


Figure 8080:2. Detail of porewater extraction cylinder. (For dimensions in centimeters, multiply dimensions in inches by 2.54.) Source: CARR, R.S. & D.C. CHAPMAN. 1995. Comparison of methods for conducting marine and estuarine sediment porewater toxicity tests. I. Extraction, storage and handling techniques. *Arch. Environ. Contam. Toxicol.* 28:29.

all methods.¹³ Toxicity tests with echinoderm gametes and embryos have been conducted to compare the toxicity of pore water obtained by various extraction techniques.¹⁴ These studies suggest that centrifugation minimizes loss of nonpolar contaminants. Loss of metals is comparable among the various extraction methods. Centrifugation is preferable to filtration for removal of particulates because it minimizes adsorptive loss of contaminants.

Sandy sediments do not compact appreciably during centrifugation, making pore water recovery difficult, whereas the pneumatic extraction method (Figures 8080:1 and 2) is particularly effective.¹¹ The vacuum method is least expensive for small-scale projects.

Regardless of the method used for initial extraction, centrifuge extracted pore water to remove suspended particulates for fertilization and embryo development assays with echinoderms and mollusks.

1. Centrifugation

Use a centrifuge equipped with a swinging bucket-type rotor capable of spinning 100- to 1000-mL bottles at $10\,000 \times g$. Use tubes or bottles composed of glass or polycarbonate to minimize adsorption of soluble contaminants on container wall. For some

sediments it may be possible to decant the supernatant without disturbing the pellet, but for most sediments, use a pipet to transfer the supernatant to a separate container.

2. Pressurized Squeeze Extraction

The most common squeeze extraction devices use compressed air (or nitrogen) to pressurize a cylinder containing the sediment. Normally use a filter in the bottom of the cylinder to minimize introduction of sediment into the porewater sample. Some filters (e.g., glass fiber filters) can adsorb a high percentage of nonpolar contaminants from solution.^{13,14} Other filter materials (e.g., polyester and nylon) are preferable. Test any part of the extraction device that contacts the pore water during the extraction process for toxicity before use. Fill extraction device with a small volume of test dilution water and, after a minimum of 8 h, test dilution water for toxicity. Soak new filters in deionized water or test dilution water with several exchanges for at least 24 h to remove any residual contaminants before use. Between samples, acid-wash the parts of the extraction devices that come in contact with the sample.

3. Vacuum Extraction

The simplest vacuum extraction system is a fused-glass air stone attached with aquarium air-line tubing to a polypropylene syringe. Apply vacuum by bracing the syringe plunger or using a vacuum pump. Modify the system with TFE* tubing and a glass syringe when loss of contaminants due to adsorption is a concern. This method is inexpensive and may retain more volatile compounds than the centrifugation or pressurized extraction methods. Vacuum methods may be more time-consuming than other extraction methods when large (>1 L) volumes are needed, particularly for fine-grained sediments. Thoroughly rinse all system components before use to remove residual toxicants.¹⁵ Determine effectiveness of the rinsing procedure by testing the toxicity of test dilution water held in the vacuum extraction system for a minimum of 8 h. Pore water extracted by the vacuum methods from sandy sediments has a higher particulate content than pore water obtained by the other methods; if the suspended particulates are not removed before testing, they may produce a response in fertilization and embryo development toxicity tests.

4. Equilibration Methods

A small-volume vessel with a membrane placed in the sediment and allowed to equilibrate with the surrounding interstitial water is the most commonly used equilibration technique for collecting pore water.^{11,16,17} The limitations to this technique are that only milliliter volumes can be obtained within a reasonable time (days). Test toxicity of components used to construct the equilibration device by soaking device in clean test dilution water or clean sediment for at least the same length of time as the longest equilibration period to be used.

*Teflon® or equivalent.

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8080 D. Toxicity Testing Procedures

1. General Procedures

Because of the difficulty in obtaining large volumes of pore water, organisms and life stages that require only small volumes are most amenable to testing with pore water. For tests requiring more than 7 d to complete, preferably use a static renewal test design to ensure acceptable water quality. Short-term toxicity tests have been used most frequently with pore water. Much of the general guidance provided in Section 8010 is applicable to testing with pore water. More specific guidance can be found in sections for particular species or groups of organisms (e.g., Sections 8510, 8610, 8710, 8720).

2. Exposure Chambers

The type of exposure chamber used depends on the test. Most porewater tests are conducted in relatively small volumes, i.e., ≤ 10 mL. Preferably cover test chambers to minimize evaporation and resulting salinity increases during the exposure period. Scintillation vials (20 mL) with polyethylene or polypropylene cap liners are ideal inexpensive disposable test chambers for many species. Avoid caps with urea-formaldehyde liners because these can be toxic. Stender dishes with ground-glass lids (20-mL capacity with 10 mL of exposure media) make excellent exposure chambers for tests that require microscopic examination of the test organisms without transferring them to another container (e.g., the *Dinophilus gyrociliatus* life-cycle test).¹

3. Organisms

Many types of organisms have been used in porewater tests. Minute species or larval forms are preferable not only for their small volume requirements, but also because they tend to be the most sensitive. Most of the studies on porewater testing have focused on marine and estuarine species.

a. Marine and estuarine species: A commercially available test system,* which detects changes in the photoluminescence of the marine bacterium *Photobacterium phosphoreum* as an end point, has been used more frequently in freshwater porewater studies²⁻⁴ than in marine or estuarine pore waters. Although the small sample size required is well suited for limited sample sizes, the sensitivity of the standard assay of this type for pore water from freshwater, estuarine, or marine sediments is low compared to those of other toxicity tests.

Algal studies with *Ulva fasciata* and *Ulva lactuca* suggest that a zoospore germination end point is as sensitive as some of the most sensitive embryological development assays used in porewater testing. This test appears to be particularly resistant to ammonia toxicity. Many algal species used in microplate procedures could easily be adapted for use with porewater samples.

Porewater toxicity testing has been conducted with the polychaete *Dinophilus gyrociliatus*.^{1,5,6} Other minute polychaetes

*Microtox® or equivalent.

such as *Ctenodrilus serratus* or *Ophryotocha* spp.^{7,8} can be tested in small volumes.

The mollusk tests used most successfully with pore water are fertilization and embryological development tests with the abalone *Haliotis refugens*. Other more common embryological development tests with oysters⁹ and clams¹⁰ could be adapted for use with porewater samples.

Most of the toxicity testing with marine and estuarine pore water has been conducted with sea urchin gametes and embryos.^{6,11,12} The species most commonly used is the sea urchin, *Arbacia punctulata*, but other species of sea urchin (e.g., *Strongylocentrotus* spp. and *Lytechinus* spp.) as well as the sand dollar (e.g., *Dendraster* spp.) also have been used successfully. Types of tests include fertilization tests, embryological development tests, and cytogenetic assay.¹³

Fish embryos and larvae of red drum *Sciaenops ocellatus* also have been used successfully in porewater testing.¹⁴

b. Freshwater species: Only a limited number of species have been used in porewater studies with fresh water. A number of studies with a commercially available system* have been reported.^{2,3,15} The freshwater amphipod *Hyaella azteca* has been used to test the toxicity of pore water from freshwater sediments.^{3,15} *Ceriodaphnia dubia* also has been used in life-cycle tests with pore water.

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8110 ALGAE

Algae are unicellular to multicellular plants that occur in fresh water, marine water, and damp terrestrial environments. All algae possess chlorophyll, the green pigment essential for photosynthesis. Algae may contain additional pigments such as fucoxanthin (brown) or phycoerythrin (red), which can mask the green color of chlorophyll. The life cycle of algae may be simple, involving cell division, or complex, involving alternation of generations. Algae are primary producers of organic matter upon which animals depend either directly or indirectly through the food chain.

Test procedures using algae are valuable for determining the primary productivity of a water and for testing the toxicity of

chemicals present in a water. Section 8111, Biostimulation (algal productivity), measures the response of a cultured species of algae to the nutritional condition of the water.

Section 8112, Phytoplankton, measures the response of an algal species to materials that interfere with its normal metabolism.

Together, the tests allow the assessment of the effects of point or nonpoint discharges in fresh and marine waters.

A third algal test measures fertilization and growth in the giant brown kelp *Macrocystis pyrifera*, which is useful in monitoring the effects of contaminants or discharges into the marine waters of the eastern Pacific Ocean.

8111 BIOSTIMULATION (ALGAL PRODUCTIVITY)*

8111 A. General Principles

1. Characteristics of Algal Assays

Algal assays consist of three steps: (a) selection and measurement of appropriate factors or conditions during the assay (e.g., biomass indicators such as measured or calculated dry weight); (b) presentation and statistical analysis of measurements; and (c) interpretation of results.

Interpretation of results involves assessment of receiving water to determine its nutritional status and its potential sensitivity to change, effects of chemical constituents on algal growth in receiving waters, effects of changes in waste treatment processes on algal growth in receiving waters, impact of nutrients in tributary waters on algal growth in lakes and confluent receiving waters, and effects of measures such as those used in lake restoration and advanced waste treatment.

The maximum standing crop and the maximum specific growth rate are responses that can be estimated from growth measurements. The maximum standing crop described in this method is proportional to the initial amount of limiting nutrient available. The maximum specific growth rate is related to concentration of rate-limiting nutrient present.

* Approved by Standard Methods Committee, 1997.

Joint Task Group: 20th Edition—Joseph C. Greene (chair), Neal E. Armstrong, Robert W. Holst, Jane Staveley Hughes, Joe M. King, Russell H. Plumb.

The algal test procedure for determining primary productivity of a water sample is based on Liebig's "Law of the Minimum," which states that growth is limited by the substance that is present in minimal quantity in respect to the need of the organism. Biostimulants are substances that increase algal growth or the potential for algal growth.

Algal species used in biostimulation tests are selected to allow for a standardized test of growth response using a well-characterized organism under standard laboratory conditions. See Sections 10010, 10200, and 10300 for methods appropriate to field studies.

Effects of various substances on maximum crop of selected algal species cultured under specified conditions are measured in this text. Results are assessed by comparing growth in the presence of selected nutrient and chelator additions to growth in controls. Experimental designs must incorporate sufficient replication to permit statistical evaluation of results.

A method for growth inhibition tests with algae has been published.¹

2. Reference

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8111 B. Planning and Evaluating Algal Assays

1. Sampling

Because water quality may vary greatly with time and point of collection, establish sampling programs to obtain representative and comparable data.

Consider all pertinent environmental factors in planning an assay, to insure that valid results and conclusions are obtained. In a stratified lake or impoundment, collect only depth-integrated (composite) euphotic zone samples. In most cases, the euphotic zone is defined as the depth to which at least 1% of the surface light is available. For euphotic zone depths of more than 8 m, subsample at least at the surface and at each 3-m depth interval. Likewise, for euphotic zones of less than 8 m, sample at least at the surface and at 2-m intervals. Composite equal-volume depth samples in a suitable nonmetallic container, mix thoroughly, and subsample for algal assay and chemical and biological analysis, including indigenous algal biomass and identification.¹

Transect lines are helpful in sampling. Samples from a transect can be taken from predetermined euphotic zones. Representative river samples can be identified by specific conductance measurements that show the homogeneity of the sampling transect. In rivers and streams, useful information may be obtained by taking

samples upstream and downstream from suspected pollutant sources or confluent tributaries.¹

The nutrient content of natural waters and wastewaters often varies greatly with time; variation may be seasonal, or even hourly in wastewaters. When sampling, consider and minimize effects of these variations.

2. Test Variables

Deficiency of any essential nutrient may limit algal growth, but tests are made for those few nutrients most likely to be growth-limiting (nitrogen, phosphorus, trace elements). Measurement of the algal growth potential of water distinguishes between the nutrients in the sample (as determined by chemical analysis) and nutrient forms that are actually available for algal growth.¹

To evaluate the potential effect of a substance on receiving waters, consider the following factors: amount and distribution, chemical and/or physical nature, fate and persistence, pathways by which it will reach the receiving water, dilution by the receiving body, and selection of appropriate test water.^{1,2}

When the algal assay is used to measure stimulation of growth by a given effluent, include the following in the overall evalua-

tion: effluent quality, growth measurements and test organisms, concentration of growth-limiting nutrient, and potential nutrient concentration and changes in availability.

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8111 C. Apparatus

1. Sampling and Sample Preparation

- a. *Sampler*, nonmetallic.
- b. *Sample bottles*, borosilicate glass, linear polyethylene, polycarbonate, or polypropylene, capable of being autoclaved.
- c. *Membrane filter apparatus*, for use with 47-mm or 104-mm prefilters (e.g., glass fiber filter) and 0.45- μ m-porosity filters.
- d. *Autoclave or pressure cooker*, capable of producing 108 kPa at 121°C.

2. Culturing and Incubation

- a. *Culture vessels*: Use erlenmeyer flasks of good-quality borosilicate glass. When trace nutrients are being studied, use special glassware made of high-silica glass or polycarbonate. While flask size is not critical, the surface-to-volume ratios of the growth medium are, because of CO₂ limitation. Use the following:

- 25 mL sample in 125-mL flask
- 50 mL sample in 250-mL flask
- 100 mL sample in 500-mL flask

- b. *Culture closures*: Use demonstrably nontoxic foam plugs,* loose-fitting aluminum foil, or inverted beakers to permit some gas exchange and prevent contamination. Determine for each batch of closures whether that batch has any significant effect on maximum specific growth rate and/or maximum standing crop.
- c. *Constant-temperature room*: Provide constant-temperature room, or equivalent incubator, capable of maintaining temperature of 18 $\pm$ 2°C (marine) to 24 $\pm$ 2°C (freshwater).
- d. *Illumination*: Use "cool-white" fluorescent lighting to provide 4304 lux $\pm$ 10% or 2152 lux $\pm$ 10% measured adjacent to the flask at the liquid level with closure in place.
- e. *Light measurement device*: Calibrate device against a standard light source or light meter.

3. Other Apparatus¹

- a. *Analytical balance* capable of weighing 100 g with a precision of $\pm$ 0.1 mg.

* Gaymar white, polyurethane foam plugs, VWR Scientific or Gaymar Industries, Inc., 701 Seneca St., Buffalo, NY 14210, or demonstrably nontoxic equivalent.

- b. Electronic particle (cell) counter.
- c. Fluorometer, suitable for chlorophyll *a*.
- d. Microscope and illuminator, good quality, general purpose.
- e. Hemocytometer or plankton counting slide.
- f. Shaker table, capable of 100 oscillations/min.
- g. pH meter to measure to ± 0.1 pH unit.
- h. Dry-heat oven capable of operating at up to 120°C.
- i. Centrifuge capable of a relative centrifugal force of at least $1000 \times g$.

8111 D. Sample Handling

1. Sampling Procedure

Use a nonmetallic water sampler and autoclavable storage container. Leave a minimum of air space in the transport container and keep it in the dark at 0 to 4°C.

2. Removal of Indigenous Algae

To use unialgal test species, "remove" indigenous algae before assay by autoclaving and filtering. Always prepare sample as soon as possible (within 24 h) after collection.

Use autoclaving followed by filtration to determine amount of algal biomass that can be grown from all bioavailable nutrients in the water, including those contained in filterable organisms. Autoclave freshwater samples at 108 kPa and 121°C for 30 min or 10 min/L of sample, whichever is longer. Pasteurize marine or estuarine samples for 4 h at 60°C. After autoclaving and cooling to room temperature, equilibrate sample by bubbling with a 1% mixture of carbon dioxide in air for at least 2 min/L. This will restore carbon dioxide lost during autoclaving and lower pH to its original level (usually it will rise on autoclaving). In some instances, waters with total hardness greater than 150 mg/L will lose calcium and phosphorus during autoclaving. The precipitate

j. Desiccator.

4. Reference

1. MILLER, W.E., J.C. GREENE & T. SHIROYAMA. 1978. The *Selenastrum capricornutum* Printz Algal Assay Bottle Test: Experimental Design, Application, and Data Interpretation Protocol. EPA-600/9-78-018, U.S. Environmental Protection Agency, Environmental Research Lab, Corvallis, Ore.

may be resistant to resolubilization by the addition of carbon dioxide and air. In waters containing high levels of hardness and alkalinity the pH may not increase during autoclaving. Filter carbon-dioxide-equilibrated sample through pre-filter, if necessary, followed by a 0.45- μ m membrane filter¹.

3. Storage

Changes occur in water samples during storage regardless of storage conditions. The extent and nature of these changes is not well known. Therefore, keep storage duration to a minimum after sample preparation. Store samples in full containers with no air space. Before sample preparation, store samples in the dark at 0 to 4°C. If prolonged storage is anticipated, prepare sample first and then store in the dark at 0 to 4°C.

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8111 E. Synthetic Algal Culture Medium

See Section 8010E.4c1).

8111 F. Inoculum

1. Recommended Test Algae

The following selected species are used primarily in the United States, Canada, and northern Europe. The tests are probably valid for other species worldwide but would require validation testing. If diatoms are the selected test species, silica must be added to the synthetic algal culture medium.

a. Freshwater algae:

Selenastrum capricornutum Printz (see Section 10900, Plate 1A:G).

b. Marine algae:

1) *Dunaliella tertiolecta* Butcher (DUN Clone) Woods Hole Oceanographic Institution.

2) *Thalassiosira pseudonana* (Hasle and Heimdal) (CN Clone) (old *Cyclotella nana*) Univ. Rhode Island. Do not shake. (See Section 10900, Plate 1B:T; Plates 29, 31.)

3) *Skeletonema costatum* (Greville) Cleve. (See Section 10900, Plate 1B:W; Plate 35.)

2. Sources of Test Algae

Obtain algal cultures from recognized sources.* After receipt of cultures, check identity and purity.

3. Maintaining Stock Cultures

a. *Medium*: See Section 8010E.4c1).

b. *Incubation conditions*:

1) Freshwater species—Temperature $24 \pm 2^\circ\text{C}$ under continuous cool-white fluorescent lighting at 4304 lux $\pm 10\%$ for *S. capricornutum*; shake at 110 oscillations/min.

2) Marine species—Temperature $18 \pm 2^\circ\text{C}$ under continuous cool-white fluorescent lighting at 4304 lux $\pm 10\%$ for *D. tertiolecta* (shake at 110 oscillations/min) and for *T. pseudonana* (do not shake but swirl daily). Higher temperatures (up to 24°C) may be justified for appropriate test species used in the Gulf of Mexico and other warm-water marine systems. If other species are used, always relate growth of those species to *D. tertiolecta* to insure comparability.

c. *First stock transfer*: Upon receipt of inoculum species, transfer a portion to the algal culture medium. (Example: 1 mL of inoculum in 50 mL in a 125-mL erlenmeyer flask).

d. *Subsequent stock transfers*: Make a new stock transfer, using aseptic technique, as the first operation on opening a stock culture. The volume transferred is not critical so long as enough cells are included to overcome significant growth lag. Make weekly stock transfers to provide a continuing supply of “healthy” cells. Check algal cultures microscopically to insure that the stock cultures remain unialgal.

e. *Age of inoculum*: Use cultures 1 to 3 weeks old as a source of inoculum. For *Selenastrum* and *Dunaliella*, a 5- to 7-d incubation often is sufficient to provide enough cells.

4. Preparing Inoculum

Centrifuge stock culture and discard supernatant. Resuspend sedimented cells in an appropriate volume of glass-distilled water containing 15 mg NaHCO_3/L for freshwater species and artificial seawater minus nutrients for marine species [Section 8010E.4c1); Table 8010:II] diluted to appropriate salinity, and again centrifuge. Resuspend sedimented algae in the proper solution and use as the inoculum.

5. Amount of Inoculum

Count cells suspended in the prepared inoculum and pipet into the test water to give a starting cell concentration as follows:

<i>S. capricornutum</i>	10^3 cells/mL
<i>D. tertiolecta</i>	10^3 cells/mL

Calculate volume of transfer to result in the above concentrations in the test flasks (e.g., for *S. capricornutum*, if there are 5×10^5 cells/mL in stock culture, transfer 0.2 mL/100 mL test water).

* American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 (phone: 800-638-6597; e-mail: sales@atcc.org); UTEX Culture Collection of Algae, Department of Botany, University of Texas at Austin, TX 78713-7640 (internet: www.botany.utexas.edu); Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), McKown Point, West Boothbay Harbor, ME 04575 (phone: 207-633-9630; e-mail: ccmp@bigelow.org).

8111 G. Test Conditions and Procedures

1. Temperature

Keep temperature at $18 \pm 2^\circ\text{C}$ for marine species and $24 \pm 2^\circ\text{C}$ for freshwater species.

2. Illumination

See Section 8111F.3b. Measure light intensity adjacent to the flask at the liquid level.

3. Procedure

a. *Preparation of glassware*: Wash all glassware with detergent (nonphosphate or sodium carbonate) and rinse thoroughly with tap water. Then rinse with a warm 10% (v/v) solution of reagent-grade HCl. Fill vials and centrifuge tubes with 10% HCl. Fill all containers to about one-tenth capacity with HCl solution and swirl to bathe entire inner surface. After HCl rinse, rinse

glassware five times with tap water, then five times with deionized water. An automatic laboratory glassware washer may be used and is the preferred method. The acid-washed glassware should be neutralized with a saturated solution of Na_2CO_3 prior to washing in an automatic washer.

If an electronic particle counter will be used, add a final rinse of deionized water that has been filtered through a 0.22- μm filter.

Dry clean glassware at 105°C in an oven and store either in closed cabinets or on open shelves with tops covered with aluminum foil.

Before use, autoclave culture flasks covered with aluminum foil at 108 kPa for 15 min. After autoclaving, prerinse flasks with culture medium and invert on absorbent paper for 20 to 30 min to drain. Close culture flasks with foam plugs.

Use disposable pipets to minimize possibility of contamination.

b. *pH control*: To insure the availability of CO_2 , keep the pH below 8.5 by using optimum surface-to-volume ratios (Sec-

tion 8111C.2), continuously shaking the flask (approximately 100 oscillations/min).

c. *Growth measurement*: Describe the growth of a test alga in the bottle test by maximum standing crop. Generally, these measurements can be made on Days 3, 5, 7, 10, 12, and 14 if a growth curve is desired. If determination of maximum standing crop is the goal, count only on Days 12 and 14.

Maximum standing crop—Maximum standing crop in any flask is defined as the maximum algal biomass achieved during incubation. For practical purposes, it may be assumed that the maximum standing crop has been achieved when the increase in biomass is less than 5%/d. The maximum standing crop usually is achieved in the algal assay test after 12 to 14 d of incubation.

After attaining the maximum standing crop, determine the biomass and relate to the dry weight gravimetrically. Although not described here, an alternative growth measurement, the maximum specific growth rate, can be measured early in the bottle test. The maximum specific growth rate (μ_{max}) for an individual flask is the largest specific growth rate (μ) occurring at any time during the incubation.

The specific growth rate, μ , is defined by:

$$\mu = \frac{\ln (X_2/X_1)}{t_2 - t_1}$$

where:

X_2 = biomass concentration at end of selected time interval,

X_1 = biomass concentration at beginning of selected time interval, and

$t_2 - t_1$ = time interval of selected time period, d.

4. Biomass Monitoring

Several methods may be used, but the selected measurements should be related to dry weight.

a. *Dry weight*: Use either the aluminum dish or membrane filter method. To use the first, centrifuge a suitable portion of algal suspension, wash sedimented cells three times in distilled water, transfer to tared crucibles or aluminum cups, dry overnight in a hot-air oven at 105°C, cool to room temperature in a desiccator jar, and weigh.

For the membrane filter method, rinse each filter with 50 mL deionized water and place in folded sheets of paper or on an aluminum weighing dish on which identification codes have been written. Dry overnight in a hot-air oven at 60°C, cool to

room temperature in a desiccator jar, and determine tare weight. Filter a suitable measured portion of algal suspension through a tared 0.45- μ m-pore-diameter membrane filter under a vacuum of 51 kPa. Use ≤ 50 mL as the cell density dictates. Rinse filter funnel with 50 mL deionized water using a wash bottle and let rinsings pass through filter. Dry in an oven for several hours at 60°C, cool in a desiccator, and weigh.

b. *Electronic particle counting*: Suspend *S. capricornutum* cells in a 1% NaCl electrolyte solution in a ratio of 1.0 mL cell suspension to 9 mL of 0.22- μ m-filtered saline (10:1 dilution). Pass the resulting suspension through a 100- μ m-diam aperture. Each cell that passes through the aperture causes a voltage drop proportional to its displaced electrolyte volume, which is recorded as a count. A knowledge of both the number of particles (cells) counted per unit volume of sample (usually 0.5 mL) and the mean particle (cell) volume displaced allows changes in cell biomass (in microliters per liter) to be calculated. Equations that can accurately relate volume to dry weight must be developed by each laboratory.

c. *Chlorophyll*: All algae contain chlorophyll and measuring this pigment can yield some insight into the relative amount of algal biomass present. To measure chlorophyll by fluorescence, swirl test flask to suspend cells. Pipet a portion of cell suspension (5 to 6 mL minimum) into a cuvette and read fluorescence. Zero fluorometer with a distilled water blank before each sample reading.

d. *Direct microscopic counting*: Use a hemocytometer or plankton counting cell (Section 10200F.2). For filamentous algae break up the algal filaments by using a syringe, an ultrasonic bath, a high-speed blender, or vigorous stirring with glass beads. Each of these techniques has drawbacks, but expelling the sample forcefully through a syringe against the inside of the flask is most satisfactory. Other methods of biomass measurement such as dry weight, absorbance, or chlorophyll fluorescence are more precise than cell counts for growth assessment of filamentous algae.

5. Bibliography

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8111 H. Effect of Additions

1. Procedures

The quantity of cells produced in a given medium is limited by the concentration of nutrient present in the lowest relative quantity with respect to that required by the organism. If a quantity of the limiting nutrient is added to the medium, cell production increases until this additional supply is depleted or until some other nutrient becomes limiting. Additions of substances other than that which is

limiting would yield no increase in cell production. Nutrient and chelator additions may be made singly or in combination and the growth response compared to that of untreated controls to identify those substances that limit growth. The selection of additives, e.g., nitrogen, phosphorus, iron, EDTA, wastewater effluents, will depend on the requirements of the test.

In all cases, keep volume of added nutrient or chelator solution as small as possible, but make it large enough to yield a poten-

tially measurable response. Relate the concentrations of additions to nutrient levels in the sample. To assess the effect of nutrient and chelator additions, compare treated sample to an untreated control. For highly productive controls, flask-to-flask variations may be high and might mask the effect of small additions of the limiting nutrient.

It is sometimes necessary to check the test water for the presence of toxic constituents. To do this, treat the sample with an appropriate dilution of the complete synthetic medium. If no growth, or less than expected growth, occurs, toxic materials are suspected. In some situations, sample dilution or addition of a chelating agent will eliminate toxic effects.

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8111 I. Data Analysis and Interpretation

1. Reporting Requirements

The fundamental measure used in the algal assay to determine biostimulation is the amount of suspended solids (dry weight) produced and determined gravimetrically. Other biomass indicators may be used, but all results must include experimentally determined conversion factors and the dry weight of suspended solids. Use several biomass indicators whenever possible, because biomass indicators respond differently to any given nutrient-limiting condition.

Report results of addition assays with results from two types of reference samples: the assay reference medium and untreated water samples. Report results of assays as maximum standing crop (with time at which it was reached).

Determine the available concentration of growth-limiting nutrient by comparing maximum standing crop in an untreated sample with a maximum standing crop in reference medium.

To determine the nutrients that limit standing crop by single-nutrient additions, treat a number of replicate flasks with single nutrients, determine the maximum standing crop for each flask, and compare the averages by Student's *t* test or other appropriate statistical tests.

To identify standing-crop-limiting nutrients by multiple-nutrient tests, make analysis-of-variance calculations. Account for

possible interaction between difference nutrients by using factorial analysis.¹

Report maximum standing crop with confidence interval. Base the calculation of confidence interval for the average values on at least five samples. Consequently, make a minimum of five replications when an unfamiliar source water is first analyzed. Use these results to calculate the standard deviation. For subsequent samples from the same source use only three replicates and report with the confidence interval established for that source water.

The overall evaluation of assay results consists of first determining whether a result is significant when considered as a laboratory measurement. Several methods are available, such as the Student's *t* test and analysis-of-variance techniques.

The second part of the evaluation is the correlation of laboratory assay results to effects observed or predicted in the field. Specific guidelines are not yet available, but note the general considerations in Section 8111B.

2. Reference

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8112 PHYTOPLANKTON*

8112 A. Introduction

The phytoplankton are primary producers in the aquatic community and, as such, are at the base of aquatic food chains. Because of this, they must be tested in bioassays that predict and determine the potential effects of a substance on the aquatic

environment. The same general principles and techniques used in determining biostimulation (Section 8111) are used to determine toxicity to phytoplankton. The procedure applies to fresh water, estuarine, and marine phytoplankton. See Sections 10200D through H and 10300D for additional information on phytoplankton.

* Approved by Standard Methods Committee, 2000.

Joint Task Group: Lenore S. Clesceri and Donald J. Reish (co-chairs).

8112 B. Inoculum

In addition to the marine or estuarine algae listed in 8111F.1b, *Monochrysis lutheri* Droop may be used. Maintain the test species in full-strength media [Section 8010E.4c1) and Tables 8010:

III and 8010:IV.A and B]. Test species must be in the logarithmic growth phase; therefore, transfer them to fresh culture medium every 4 to 5 d.

8112 C. Test Conditions and Procedures

1. Maximum Specific Growth Rate

The maximum specific growth rate (μ_{max}) can be estimated from growth measurements. It is related to the concentration of rate-limiting nutrient available. The μ_{max} is the greatest specific growth rate (μ) occurring at any time during an incubation. The specific growth rate is defined by:

$$\mu = \frac{\ln(X_2/X_1)}{t_2 - t_1}$$

where:

X_2 = biomass concentration at end of selected time interval,

X_1 = biomass concentration at beginning of selected time interval, and

$t_2 - t_1$ = time interval of selected time period, d.

Add test material to test vessels to give desired concentrations. Prepare triplicate vessels for each concentration. Use dilutions of culture medium to simulate chemical conditions of specific receiving waters. For optimum surface-to-volume ratios, see Section 8111C.2a.

The maximum specific growth rate (μ_{max}) occurs during the logarithmic phase of growth, usually between Day 0 and Day 5. Therefore, measure biomass at least daily during the first 5 d of incubation. Indirect measurements of biomass, such as chlorophyll *a* or cell numbers, usually will be required because accurate gravimetric measurements at low cell densities are difficult. See Section 8111G.3c and 8111G.4 for methods.

Test a geometric series of concentrations initially (see Section 8010F.3b). After this preliminary test, progressively bisect intervals on a logarithmic scale. Narrow the range of test concentrations to determine the concentration that reduces the maximum specific growth rate (μ_{max}) to 50% that of the control. This requires that two of the concentrations tested fall on each side of the concentration that inhibited (μ_{max}) to 50% (see Section 8010G).

Compare the maximum specific growth rate (μ_{max}) to that obtained in the synthetic freshwater or artificial seawater culture medium. Regional and seasonal variations in quality make natural waters unsuitable as standard test media for comparative toxicity tests. Therefore, use a synthetic freshwater medium and/or artificial seawater. Add various concentrations of toxicants to the culture medium in triplicate and inoculate with test species.

2. Other Tests

For other types of tests, such as those to determine effluent requirements or compliance with water quality standards, take dilution water from the receiving body near the outfall but outside its influence. Remove undesirable organisms before making growth rate tests with selected sensitive species (Section 8111D). Determine maximum specific growth rates (μ_{max}) in test vessels and compare with controls and EC50s based on percent of growth reduction. An alternative approach that provides a number that may relate to natural conditions should be reviewed.¹

3. Reference

1. MILLER, W.E., J.C. GREENE & T. SHIROYAMA. 1978. The *Selenastrum capricornutum* Printz Algal Assay Bottle Test. U.S. Environmental Protection Agency Rep. EPA-600/9-78-018, National Technical Information Serv., U.S. Dep. Commerce, Springfield, Va.

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8113 MARINE MACROALGAE*

8113 A. Introduction

1. Occurrence and Importance

Marine macroalgae are important biological components of the oceans of the world and are found in coastal and intertidal zones down to the depths of light penetration sufficient for photosynthesis. They are classified in one of three phyla based, to a large extent, on color derived from the major photosynthetic pigments present. Members of Chlorophyta (green algae) contain chlorophyll *a* and *b* and β -carotene. The red algae (Rhodophyta) contain phycoerythrin, an accessory pigment that provides the reddish color observed in most species of this phylum. The brown algae (Phaeophyta) contain the brown accessory pigment fucoxanthin. Within this phylum are the most structurally complex of all the algae, the laminarians (Order Laminariales). Laminarians include the large, canopy-forming kelps of the world such as the giant kelp, *Macrocystis pyrifera*.

The giant kelp, *Macrocystis pyrifera*, (see Section 10900, Plate 2A:E) forms extensive coastal submarine forests that provide food, habitat, and shelter for numerous species.¹ The giant kelp also is an important economic resource that is harvested commercially to obtain alginic acid, a thickening agent used in foods, cosmetics, culture media, and other products. The economic and ecological importance of *Macrocystis pyrifera* as well

as its abundance make it an attractive test species for monitoring coastal water quality. Indeed, test methods using the early life stages of giant kelp have been developed and found to be sensitive to many toxicants found in the coastal environment.²⁻⁴ The *Macrocystis pyrifera* method has been used successfully for monitoring and compliance determination of National Pollution Discharge Elimination System (NPDES) permits in California and other regions along the Pacific Coast of North America since 1990.⁵

2. References

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2. JAMES, D.E., S.L. MANLEY, M.C. CARTER & W.J. NORTH. 1987. Effects of PCBs and hydrazine in life processes in microscopic stages of selected brown seaweeds. *Hydrobiologia* 151/152:411.
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* Approved by Standard Methods Committee, 1999.
Joint Task Group: Joseph R. Gully (chair), Robert W. Holst, Donald J. Reish.

8113 B. Selecting and Preparing *Macrocystis pyrifera* Sporophylls

1. Life Cycle

The life cycle of *Macrocystis pyrifera*, like that of all laminarians, alternates between a diploid sporophyte and a haploid gametophyte generation (Figure 8113:1). The conspicuous adult sporophyte produces haploid, dioecious, biflagellate zoospores in dark patches (sori) found on reproductive blades (sporophylls) near the base of the plant throughout the year. The zoospores are released and settle on an appropriate substrate where they germinate and develop into microscopic gametophytes. Flagellated male gametes are produced within the antheridia of the male gametophytes and released to fertilize eggs produced within oogonia on the female gametophyte. The fertilized eggs develop into young sporophytes in 12 to 15 d.

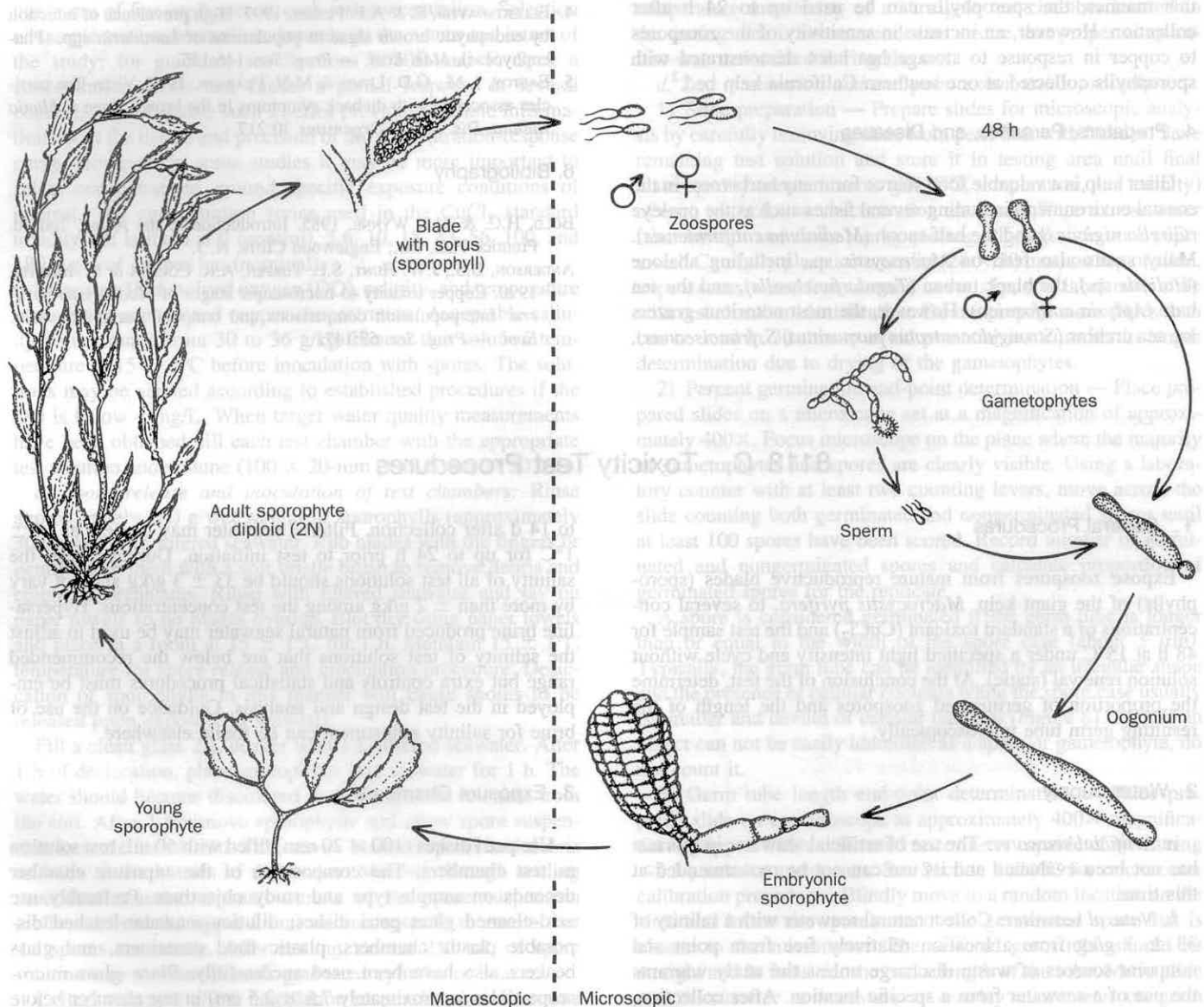


Figure 8113:1. The life cycle of the giant kelp, *Macrocystis pyrifera*.

2. Occurrence

Macrocystis pyrifera forests are found subtidally along the Pacific Coast of North America from Central California south to Baja California. Substantial populations also are found along the west coast of South America from Peru south and off the coasts of New Zealand, Tasmania, and the islands of the subantarctic.¹

3. Collection and Maintenance of Test Organisms

Zoospores of *Macrocystis pyrifera* are obtained by subtidal collection (usually by SCUBA divers) of reproductive blades called sporophylls. The sporophylls are found near the base of

the plant, usually lack air bladders, are thinner than vegetative blades, and have sori that extend to near the margin of the sporophyll. The sori contain the mature and developing zoospores. Unless contrary to the purpose of the study, collect sporophylls from several plants and in a region relatively free from point and nonpoint sources of water discharge and below the thermocline, if present. Check local regulations and collection permit requirements before obtaining sporophylls.

Once sporophylls have been collected, keep them damp but avoid immersion in seawater because this will cause the zoospores to be released. Keep blades out of direct sunlight to avoid damage to the zoospores from ultraviolet radiation. Clean the sporophylls by gently rubbing with fingers or soft bristle brush and rinse with seawater filtered through 0.22- μ m filter. Layer the sporophylls between paper towels to prevent any blades from overlapping. Keep temperature of the sporophylls between 9 and 12°C during transport and/or shipment to test facility using newspaper-insulated chemical ice packs and a cooler. Stored in this manner, the sporophylls can be used up to 24 h after collection. However, an increase in sensitivity of the zoospores to copper in response to storage has been demonstrated with sporophylls collected at one southern California kelp bed.²

4. Predators, Parasites, and Diseases

Giant kelp is a valuable food source for many herbivores in the coastal environment, including several fishes such as the opaleye (*Girella nigricans*) and the half-moon (*Medialuna californiensis*). Many snails also feed on *Macrocystis* sp., including abalone (*Haliotis* sp.), the black turban (*Tegula funebris*), and the sea hare (*Aplysia californica*). However, the most notorious grazers are sea urchins (*Strongylocentrotus purpuratus*, *S. franciscanus*),

which can cause extensive damage to giant kelp forests when the population explodes because of a lack of predation.³

Certain bryozoans, particularly *Membranipora membranacea*, use blades of giant kelp as a substrate for attachment; do not use encrusted sporophylls for toxicity tests. Morphological deformities associated with endophytic algae⁴ and epidemic diebacks from infection by virus-like particles⁵ have been documented in other species of kelp. Collect sporophylls for use in toxicity tests only from plants that appear healthy and free from deformities.

5. References

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8113 C. Toxicity Test Procedures

1. General Procedures

Expose zoospores from mature reproductive blades (sporophylls) of the giant kelp, *Macrocystis pyrifera*, to several concentrations of a standard toxicant (CuCl_2) and the test sample for 48 h at 15°C under a specified light intensity and cycle without solution renewal (static). At the conclusion of the test, determine the proportion of germinated zoospores and the length of the resulting germ tube microscopically.

2. Water Supply

a. Artificial seawater: The use of artificial seawater in this test has not been evaluated and its use can not be recommended at this time.

b. Natural seawater: Collect natural seawater with a salinity of 33 ± 3 g/kg from a location relatively free from point and nonpoint sources of waste discharge unless the study warrants the use of a seawater from a specific location. After collection, filter using a 0.45- μ m membrane filter and store in the dark at $4 \pm 1^\circ\text{C}$ until used. Seawater held in this manner may be used up

to 14 d after collection. Filtered seawater may be held at $15 \pm 1^\circ\text{C}$ for up to 24 h prior to test initiation. During a test, the salinity of all test solutions should be 33 ± 3 g/kg and not vary by more than ± 2 g/kg among the test concentrations. Hypersaline brine produced from natural seawater may be used to adjust the salinity of test solutions that are below the recommended range but extra controls and statistical procedures must be employed in the test design and analysis. Guidance on the use of brine for salinity adjustment can be found elsewhere.¹

3. Exposure Chambers

Use petri dishes (100 $\times$ 20 mm) filled with 50 mL test solution as test chambers. The composition of the exposure chamber depends on sample type and study objectives. Preferably use acid-cleaned glass petri dishes; dilution-seawater-leached disposable plastic chambers, plastic food containers, and glass beakers also have been used successfully. Place glass microscope slides (approximately 7.6 $\times$ 2.5 cm) in test chamber before adding test solution and spores. The slides serve as a substrate for spore settlement and gametophyte development that can be

removed and analyzed microscopically at the end of the test. This testing system is not suitable for volatile compounds.

4. Conducting the Toxicity Test

a. Preparation of test chambers for inoculation: Label test chambers in a way that will facilitate blind analysis of the biological end points at the conclusion of the exposure. Place test chambers randomly in testing area to account for subtle differences in light intensity and temperature. Measure light intensity at each test chamber using a planar light meter and adjust light fluence rate and/or position of chambers as required to obtain a fluence rate of $50 \pm 10 \mu\text{Einsteins/m}^2/\text{s}$ (2000 to 3010 lux; 186 to 280 ft-c). Set the light cycle in testing area for 16 h light and 8 h dark for the duration of the test.

Prepare volumes of test solution needed to provide solution for test chambers and water quality/chemistry measurements. Use minimum of five replicates at each test concentration. Selection of sample test concentrations depends on the nature and intent of the study; for guidance see Sections 8010D and F. Seek a concentration series that causes a partial response at several concentrations because such a series provides valuable information about the nature and precision of the concentration-response curve. However, in some studies it may be more important to select concentrations around specific exposure conditions of interest. The concentration series used in the CuCl_2 standard toxicity test is fixed at 0 (control), 5.6, 10, 18, 32, 56, 100, and 180 $\mu\text{g/L}$ of copper ion (nominal).

Measure pH, dissolved oxygen (DO), salinity, and temperature of each test sample and copper concentration. Acceptable salinity values range from 30 to 36 g/kg. Ensure that solution temperature is $15 \pm 1^\circ\text{C}$ before inoculation with spores. The solutions may be aerated according to established procedures if the DO is below 4 mg/L. When target water quality measurements have been obtained, fill each test chamber with the appropriate test solution and volume ($100 \times 20\text{-mm}$ petri dishes = 50 mL).

b. Spore release and inoculation of test chambers: Rinse approximately 100 g wet weight of sporophylls (approximately 30 blades) with filtered seawater. Rub blades with the fingers or gently scrub them with a soft bristle brush to remove debris and epiphytic organisms. Rinse with filtered seawater and lay on paper towels so no blades overlap. Blot dry using paper towels and place in a room at $15 \pm 1^\circ\text{C}$ for 1 h. Maintain $15 \pm 1^\circ\text{C}$ temperature for spore release and inoculation procedure. Desiccation of sporophylls in this manner will cause spores to be released upon exposure to seawater.

Fill a clean glass 2-L beaker with 1 L filtered seawater. After 1 h of desiccation, place sporophylls into seawater for 1 h. The water should become discolored as the spores are released from the sori. After 1 h remove sporophylls and allow spore suspension to settle for 30 min. This allows inactive spores and debris to settle to the bottom of the beaker. Midway through settling procedure examine a small sample of the spore suspension (collected from the upper 2 cm) microscopically to ensure that the spores are motile. After settling, decant 250 mL of spore solution into a clean 250-mL graduated cylinder or beaker.

Collect a sample of the inoculation suspension and determine density of the spores using a hemacytometer. The target density of spores in the test chambers is 7500 spores/mL. Calculate

volume of inoculation suspension required to obtain target spore density using the equation:

$$V_i = (7500 \text{ spores/mL} \times V_t) / D_i$$

where:

V_i = volume of inoculation added to test chamber,

V_t = volume of test suspension in test chamber, and

D_i = spore density of inoculation suspension.

Typically, the volume of the inoculant required to obtain 7500 spores/mL is <1% of the test volume. If greater volumes are required, the quality of the spores and consequent test results should be suspect. Inoculate each test chamber with spore suspension using a cut or wide-mouth pipet. Note the time, which represents the start of the test.

c. Duration and type of test: The exposure of the spores to test solution is static and the duration is 48 ± 3 h. Continuous temperature monitoring of a surrogate test chamber located in the testing area is recommended to ensure that proper temperature was maintained during the test.

d. Test termination and end-point examination procedures:

1) Slide preparation — Prepare slides for microscopic analysis by carefully removing slide from petri dish with forceps. Save remaining test solution and store it in testing area until final water quality measurements (pH, DO, temperature, and salinity) of each test solution concentration are made. Wipe excess water from bottom and drain top by placing edge of slide on a paper towel. Carefully place a cover slip (24×50 mm) on top of slide, wipe excess water from around cover slip, and label each slide with test chamber identification. Do not prepare more slides than can be read in 30 min to avoid potential inaccuracies in length determination due to drying of the gametophytes.

2) Percent germination end-point determination — Place prepared slides on a microscope set at a magnification of approximately $400\times$. Focus microscope on the plane where the majority of gametophytes and spores are clearly visible. Using a laboratory counter with at least two counting levers, move across the slide counting both germinated and nongerminated spores until at least 100 spores have been scored. Record number of germinated and nongerminated spores and calculate proportion of germinated spores for the replicate.

A spore is considered germinated if the germ tube is longer than or equal to the width of the spore case (about $3 \mu\text{m}$). Typically, the germ tube can be identified by an irregular shape and the presence of cellular contents while the spore case usually is circular and devoid of cellular material (Figure 8113:2). If an object can not be easily identified as a spore or gametophyte, do not count it.

3) Germ tube length end-point determination — Place prepared slide on a microscope at approximately $400\times$ magnification equipped with a calibrated micrometer capable of measuring $\leq 2.5 \mu\text{m}/\text{micrometer unit}$. See Section 10200E for micrometer calibration procedures. Blindly move to a random location on the slide and locate the spore case of the germinated spore that is closest to the micrometer. The selected germ tube should be straight and in focus for the entire length of the tube. Move slide and micrometer as necessary to overlay micrometer on germ tube. The length of the germ tube is defined as the distance from the edge of spore case at the base of the germ tube to the tip

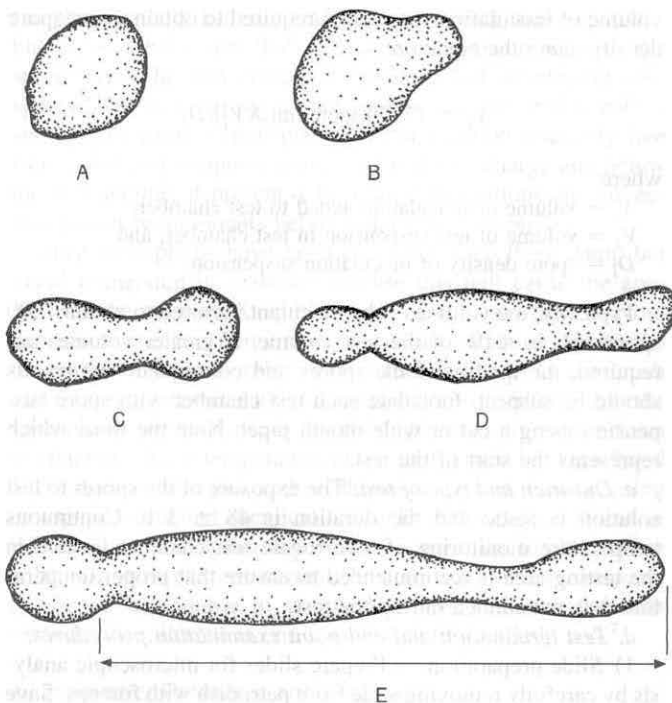


Figure 8113:2. Examples of nongerminated (A and B) and germinated (C and D) giant kelp zoospores and germ-tube-length measurement of germinated zoospores (E). Zoospores are considered germinated when the germ tube length is equal to or greater than the width of the spore case. The length of the germ tube is measured from the edge of the spore case to the tip of the germ tube.

(Figure 8113:2). Measure and record germ tube length rounded to nearest whole micrometer unit. Once germ tube length has been recorded, blindly move to another field of view and repeat

selection and measuring procedures until 10 germ tubes have been measured. If germination percentage is low (<10%), the slide may be scanned for germinated spores, measuring at the first 10 germ tubes identified. Calculate mean germ tube length in micrometer units and convert mean value into microns using the conversion factor derived from the calibration procedure. Record this value as the mean germ tube length for the replicate.

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8113 D. Data Evaluation

1. Statistical Analysis

The statistical analysis selected to evaluate the toxic nature of the sample or the reference toxicant depends on the objectives of the study and is discussed in Section 8010G.

The mean germination percentage and germ tube length in seawater controls for each test and the EC25/IC25 and mean square error (MSE) values derived from analysis of variance (ANOVA) must be calculated for the reference toxicant test to evaluate the quality of the test. If brine was added to any test concentrations, recalculate toxicant concentrations and compare response of brine controls with natural seawater controls using a *t*-test. If control responses were statistically different (two-tailed; $\alpha=0.05$), interpretation of the data may become difficult and may warrant repeating the test. If the data are used, perform

separate statistical analyses for unaltered and brine-enriched treatments using the corresponding control data.

2. Toxicity Test Evaluation

a. General considerations: The following test acceptability criteria are intended to help ensure that the test has utilized healthy kelp sporophylls and spores and adequate dilution water, and has been performed in a manner consistent with the method described above. Data derived from tests that do not meet these criteria should be considered suspect and may warrant repeating the test.

b. Negative control minimums: The purpose of negative control minimum criteria is to ensure that the spores germinated and grew to a minimal level consistent with those seen in tests using

healthy sporophylls, adequate dilution seawater, prescribed procedures, and qualified staff. The minimum mean germination percentage and germ tube length of the control replicates in the reference toxicant and sample tests should be 70% and 10 μm , respectively.

c. *Positive control response ranges*: The purpose of positive control response range criteria is to ensure that the spores' response to a standard toxicant, copper, is consistent with that observed in tests using healthy sporophylls, adequate dilution seawater, prescribed procedures, and qualified staff. The EC25 for the germination end point of the reference toxicant test should be between 18 and 158 μg Cu/L. The IC25 in the germ-tube-length end point of the reference toxicant test should be between 13 and 106 μg Cu/L.

8200 AQUATIC FLOWERING PLANTS

Aquatic flowering plants belong to phylum Spermatophyta, characterized by possession of true roots, stems, and leaves and production of seeds from flowers. The phylum contains most of

d. *Within-test variability maximums*: The purpose of within-test variability maximum criteria is to ensure that the variability of the spores' response, particularly among treatment replicates, does not exceed the variability observed in tests using healthy sporophylls, adequate dilution seawater, prescribed procedures, and qualified staff. The measure of variability used for this criterion is the mean square error (MSE) derived from ANOVA. The MSE for the proportion germinated end point of the reference toxicant test should be less than 63.3 deg or 0.019 radians for arcsin square root transformed data. The MSE for the germ-tube-length end point of the reference toxicant test should be less than 4.32 μm .

Joint Task Group: 20th Edition — Donald J. Reish (chair),

8211 DUCKWEED*

8211 A. Introduction

1. Organism Characteristics

Lemna minor L. (Figure 8211:1) (also known as common duckweed) is a small flowering aquatic macrophyte (a monocot) widely distributed in quiescent fresh water and estuaries ranging from tropical to temperate zones.¹ It is the most common species of the family Lemnaceae in the United States and many other parts of the world. It is morphologically simple, consisting only of frond and root. The frond size is approximately 2 to 4 mm and root length is up to 50 mm. The plant is colonial (up to 8 fronds), multiplies sexually and asexually, and has a growth rate far exceeding those of other flowering plants. Duckweed is a food for waterfowl and small animals, and provides food, shelter, and shade for fish and other aquatic organisms. Furthermore, it serves as a habitat for various invertebrates.

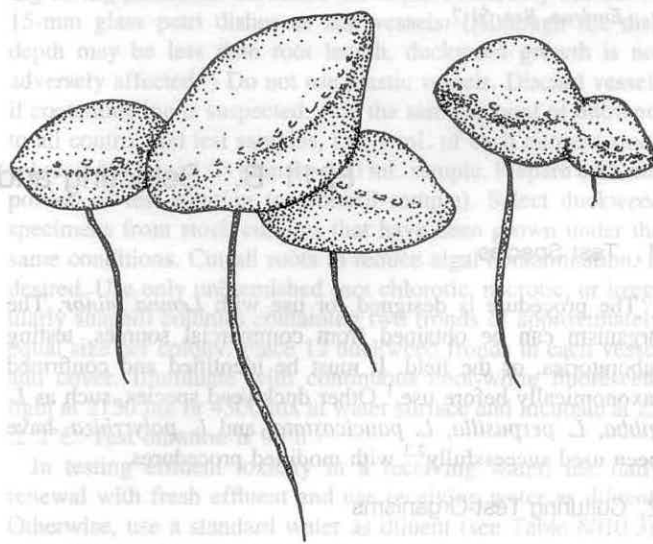


Figure 8211:1. Common duckweed: *Lemna minor*. Each plant consists of frond and root; together, plants form colony.

* Approved by Standard Methods Committee, 1997.

Joint Task Group: Wuncheng Wang (chair), James F. Fairchild, Joe M. King, Michael A. Lewis, Jerry C. Smrcek, Jane Staveley, James P. Swigert, David E. Weber.

2. Test Applications

Common duckweed is an ideal organism for testing aquatic phytotoxicity of herbicides, industrial and municipal wastewaters, and other contaminants.² Because many ambient waters and effluents are colored and/or turbid, they are difficult to test directly for toxicity by using algal testing without filtering, which can alter sample integrity. Duckweed, being floating plants, can be used to test colored or turbid samples without filtration. In addition, some samples may contain labile, volatile, or sorptive materials and require either renewal or flow-through methods. Algal testing may be inappropriate for these types of tests, whereas the duckweed toxicity test as described herein can be modified easily to apply in either method.

The duckweed toxicity test is useful, especially for determining phytotoxicity at the air-water interface where surface-active substances, oil and grease, and toxic organic compounds may be concentrated. The test also is useful for determining toxicity of metals,³ organic compounds,^{4,5} and industrial and municipal effluents⁶⁻⁹ and herbicides.¹⁰ However, as vascular monocots, duckweeds are insensitive to auxin stimulating herbicides such as 2,4-D. The test is generally described as simple, sensitive, and cost-effective.²

Common duckweed, being a floating plant, may underestimate the toxicity of a substance that adsorbs on particulate matter and precipitates during a static test. Also, substances that concentrate at the air-water interface tend to affect duckweed more than other aquatic plants, such as algae or submerged plants. Gentle shaking or stirring test vessels to increase mixing may overcome or lessen these problems. Alternatively, renewal or flow-through methods can be used.

Several standard tests using duckweed are available in the literature.^{11,12}

3. References

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12. ENVIRONMENT CANADA. 1999. Biological Test Method: Test for measuring the inhibition of growth using the freshwater macrophyte, *Lemna minor*. EPS 1/RM 37, Environment Canada, Ottawa, Ont.

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8211 B. Selecting and Preparing Test Organisms

1. Test Species

The procedure is designed for use with *Lemna minor*. The organism can be obtained from commercial sources, testing laboratories, or the field. It must be identified and confirmed taxonomically before use.¹ Other duckweed species, such as *L. gibba*, *L. perpusilla*, *L. paucicostata*, and *L. polyrrhiza*, have been used successfully^{2,3} with modified procedures.

2. Culturing Test Organisms

Acclimate a new duckweed culture to the test environment (e.g., lighting, temperature, and nutrient conditions) for at

least 2 weeks before a test. This culture grows vigorously and provides a nearly inexhaustible supply for testing under proper conditions. Grow duckweed in a 15-L culture vessel such as an aquarium or stainless steel basin. To prepare a 10-L culture solution, add 100 mL of each stock nutrient solution A, B, and C (see Table 8211:I), to deionized or other suitable water. Do not use tapwater. Use a water depth of at least 40 mm or more and provide constant cool-white fluorescent light (2150 lux to 4300 lux at the water surface). Maintain a temperature of $25 \pm 2^\circ\text{C}$. Add diluted (1/4 strength) culture solution weekly. Transfer stock culture to a freshly prepared nutrient solution monthly. Axenic culturing is unnecessary.

TABLE 8211:I. DUCKWEED NUTRIENT SOLUTION

Solution	Stock Solution Concentration	Element	Final Concentration
A:			
NaNO ₃	25.5 g/L	N	42.0 mg/L
		Na	110.0 mg/L
NaHCO ₃	15.0 g/L	C	21.4 mg/L
K ₂ HPO ₄	1.04 g/L	K	4.69 mg/L
		P	1.86 mg/L
B:			
CaCl ₂ ·2H ₂ O	4.41 g/L	Ca	12.0 mg/L
MgCl ₂	5.7 g/L	Mg	29.0 mg/L
FeCl ₃	0.096 g/L	Fe	0.33 mg/L
Na ₂ EDTA·2H ₂ O	0.3 g/L		
MnCl ₂	0.264 g/L	Mn	1.15 mg/L
C:			
MgSO ₄ ·7H ₂ O	14.7 g/L	S	19.1 mg/L
H ₃ BO ₃	0.186 g/L	B	325 µg/L
Na ₂ MoO ₄ ·2H ₂ O	7.26 mg/L	Mo	28.8 µg/L
ZnCl ₂	3.27 mg/L	Zn	15.7 µg/L
CoCl ₂	0.78 mg/L	Co	3.54 µg/L
CuCl ₂	0.009 mg/L	Cu	0.04 µg/L

NOTE:

1. Omit Na₂EDTA·2H₂O in Solution B if test samples contain toxic metals. In that case, acidify Solution B to pH 2 to prevent precipitation.
2. To prepare the duckweed nutrient solution, add 1 mL of each stock solution to 100 mL deionized water. Adjust to pH 7.5 – 8.0.

8211 C. Toxicity Test Procedure

1. General Considerations

Use static, renewal,¹ or flow-through methods.^{1,2} As a general rule, if a test solution is stable (e.g., a solution with low microbial population, high toxicity, or low volatility), use a static test. If samples are unstable, use renewal (daily renewal) or flow-through methods (see Section 8010D).

2. Preparing Test Materials

As used herein, dilution water and control water are identical to duckweed nutrient solution. For preparation of this solution, see Table 8211:I.

For preparation of toxicant solutions, see Section 8010F.2b.

3. Test Procedures and Conditions

Screening tests use a predetermined concentration (e.g., 100% effluent) to determine if a sample is toxic in comparison with the control water. If the sample is toxic, test it further using range-finding and definitive tests. In the range-finding test, examine a series of concentrations, usually at ratios of 10, e.g., 10%, 1%, 0.1%, etc.

Range-finding-test results are used as a basis for designing definitive tests. For definitive tests use five concentrations of sample in a ratio of 0.5, e.g., 10%, 5%, 2.5%, etc. Ideally, prepare a series of solutions in which the midpoint concentration

3. Diseases and Predators

Diseases, phytophagous insects, or other pests do not normally pose any problem to a duckweed culture. If the culture appears unhealthy (e.g., chlorotic or necrotic), destroy the old culture and start a new one. It is good practice to maintain several cultures isolated from each other. When epiphytic algae become a problem, use 0.5% sodium hypochlorite solution for 0.5 to 5 min, then rinse in sterile water.

4. References

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produces approximately 50% inhibitory effect, and the highest and lowest concentrations produce approximately 90 and 10% inhibitory effects.

Use four replicates. Include a negative control containing only duckweed nutrient solution as well as a positive control containing 20 mg potassium chromate (as Cr)/L. Preferably use 60- × 15-mm glass petri dishes as test vessels. (Although the dish depth may be less than root length, duckweed growth is not adversely affected.³) Do not use plastic vessels. Discard vessels if contamination is suspected. Add the same amount of nutrients to all control and test samples, i.e., 1 mL of each nutrient stock solution (A, B, and C) to make 100 mL sample. Prepare a 15-mL portion of test solution (or control sample). Select duckweed specimens from stock cultures that have been grown under the same conditions. Cut all roots to reduce algal contamination, if desired. Use only unblemished (not chlorotic, necrotic, or irregularly shaped) colonies containing two fronds of approximately equal size per colony. Place 12 duckweed fronds in each vessel and cover. Illuminate with continuous cool-white fluorescent light at 2150 lux or 4300 lux at water surface and incubate at 25 ± 2°C. Test duration is 96 h.

In testing effluent toxicity in a receiving water, use daily renewal with fresh effluent and use receiving water as diluent. Otherwise, use a standard water as diluent (see Table 8010:3). Complete frond counts daily to determine any intermediate toxic effects. In some cases, duckweed may exhibit delayed effects; it takes about 2 to 3 d for the growth rate to change when duck-

weed is transferred to a new solution. Stimulatory effects may occur in some instances.

4 Test Results

Observe duckweed plants under a lighted magnifying glass (2 × or higher) for symptoms, including chlorosis (loss of pigment/yellowing), necrosis (localized dead tissue), colony breakup, root destruction, loss of buoyancy, and gibbosity (humpback or swelling). Compare affected fronds with duckweed specimens in the control.

Count frond increase daily to provide a quantal value directly reflecting duckweed growth. To measure frond increase, count every visible, protruding bud. This nondestructive method allows repeated observations of the same test vessel.

Other methods that have been used include ^{14}C uptake, chlorophyll content, biomass, frond area, plant colony counts, total root number and root length.^{4,5} Mortality alone is of limited value.⁵

Some substances, at low concentrations, stimulate, rather than inhibit, duckweed growth. Report this effect if it is observed.

5. Statistical Analysis

Follow general procedures described in Section 8010G.

In screening tests, the key question is whether a sample is toxic or stimulatory relative to the control. Express toxicity (or stimulation) in percent inhibition (or stimulation) relative to the control

$$\% I = 100 (C - T)/C$$

where C and T are average increases in number of fronds in control and test samples, respectively.

Results of definitive tests may be graphed using linear, semi-log, or log-log plots. Typically, the concentration-effect relationship is sigmoidal. Determine IC_{50} value (the concentration causing 50% inhibitory effect) by graphical or statistical methods. The slope of dose-response relationship is toxicant-specific and thus can be valuable information.

8220 AQUATIC EMERGENT PLANTS*

8220 A. Introduction

1. Organism Characteristics

Emergent plants are important components of aquatic and wetland ecosystems. They are among the primary producers,

6. Quality Control

The negative control sample is necessary for quality control effect measurement. A test is not acceptable if more than 10% of the control specimens show adverse symptoms (8211C.4). Under normal conditions, the doubling time for duckweed is less than 2 d. If the control sample yields less than a twofold increase in fronds in 96 h, the test is not acceptable.

Use a reference chemical at a single specified concentration as a positive control. The chromate ion has been found to be an ideal reference toxicant. Its toxicity or chemical speciation is not affected by water quality and thus it can be used in natural waters, wastewaters, leachates, and the like; 20 mg/L Cr(VI) concentration causes approximately 60% inhibitory effect of duckweed growth.^{6,7} Include chromate ion as a positive control in every test.

7. References

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providing oxygen, food, and habitat for periphyton and many other organisms, including invertebrates, fish, amphibians, birds, and mammals. These plants function in nutrient cycling and in the stabilization of sediments of near-shore environments.

2. Test Applications

This method is designed to evaluate phytotoxicity of chemical substances in water on emergent plants. The method uses several

* Approved by Standard Methods Committee, 1997.

Joint Task Group: Wuncheng Wang (chair), James F. Fairchild, L. Gordon Goldsborough, Robert W. Holst, Lawrence A. Kapustka, Jerry C. Smrcek, Jane Staveley, David E. Weber.

measurement endpoints of toxic response, including seed germination, root elongation, root dry weight, and dry weight of the seedling shoot. Seed germination and seedling growth represent the first phase of plant development. Significant inhibition of this developmental phase may diminish the ability of affected plants to compete and survive in their environment.

The seed germination and seedling growth tests are simple, versatile, and useful for screening toxicity in water.¹⁻³ The tests are useful for evaluating toxicity of metals,⁴⁻⁶ organic compounds,^{5,7} and complex effluents.^{2,3} They can be conducted in fresh, sea, or brackish water, with the use of appropriate plant species. They are applicable to turbid or discolored aqueous samples.³ Sediments can be screened for toxicity by testing aqueous extracts, pore water, or whole sediment.^{2,8}

One advantage of seed germination and seedling growth tests is that seeds can be obtained in bulk quantity and stored for extended periods with minimal maintenance costs. Stored plant seeds are quiescent and resistant to environmental stress. Under favorable conditions for germination, seeds undergo rapid changes and become highly sensitive to the environment.¹ Standard water (Section 8010E) can be used as dilution water and control solution (see 8220C.2). The tests are highly desirable to complement other plant tests (e.g., algal or duckweed tests) where the potential for interaction of nutrients and adjuvants may exist.

The tests can be conducted in darkness or light with the species suggested. When both dark- and light-phase experiments are conducted, one can evaluate the effects of photodegradation on availability and toxicity of toxicants.

The tests can be performed with a relatively small volume of test solution (30 mL/vessel or less) compared to aquatic animal testing (100 to 200 mL/vessel). A small volume may be used to lower the cost of sample collection and storage; however, as the volume of test medium is lowered, there may be diminishing capacity to detect phytotoxic effects.

Other toxicity tests for aquatic emergent plants are available.^{9,10}

3. References

1. MAYER, A.M. & A. POLJAKOFF-MYBER. 1982. The Germination of Seeds, 3rd ed. Pergamon Press, Oxford, England.
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8220 B. Selecting and Preparing Test Organisms

1. Test Species

Criteria for selecting test species include availability, cost, similarity of test species to species of interest, consistent performance, and high germination percentage.¹ There are many emergent species² but only a few are readily available.^{3,4}

The following freshwater plant species have been used successfully in toxicity testing and are available from commercial vendors:*

* For example, Wildlife Nurseries, P.O. Box 2724, Oshkosh, WI 54903; Environmental Concern, P.O. Box P, St. Michaels, MD 21663; Kester's Wild Game Food Nurseries, Inc., Omro, WI 54963; or Mangelsdorf Seed Co., 1415 13th Street, St. Louis, MO 63106.

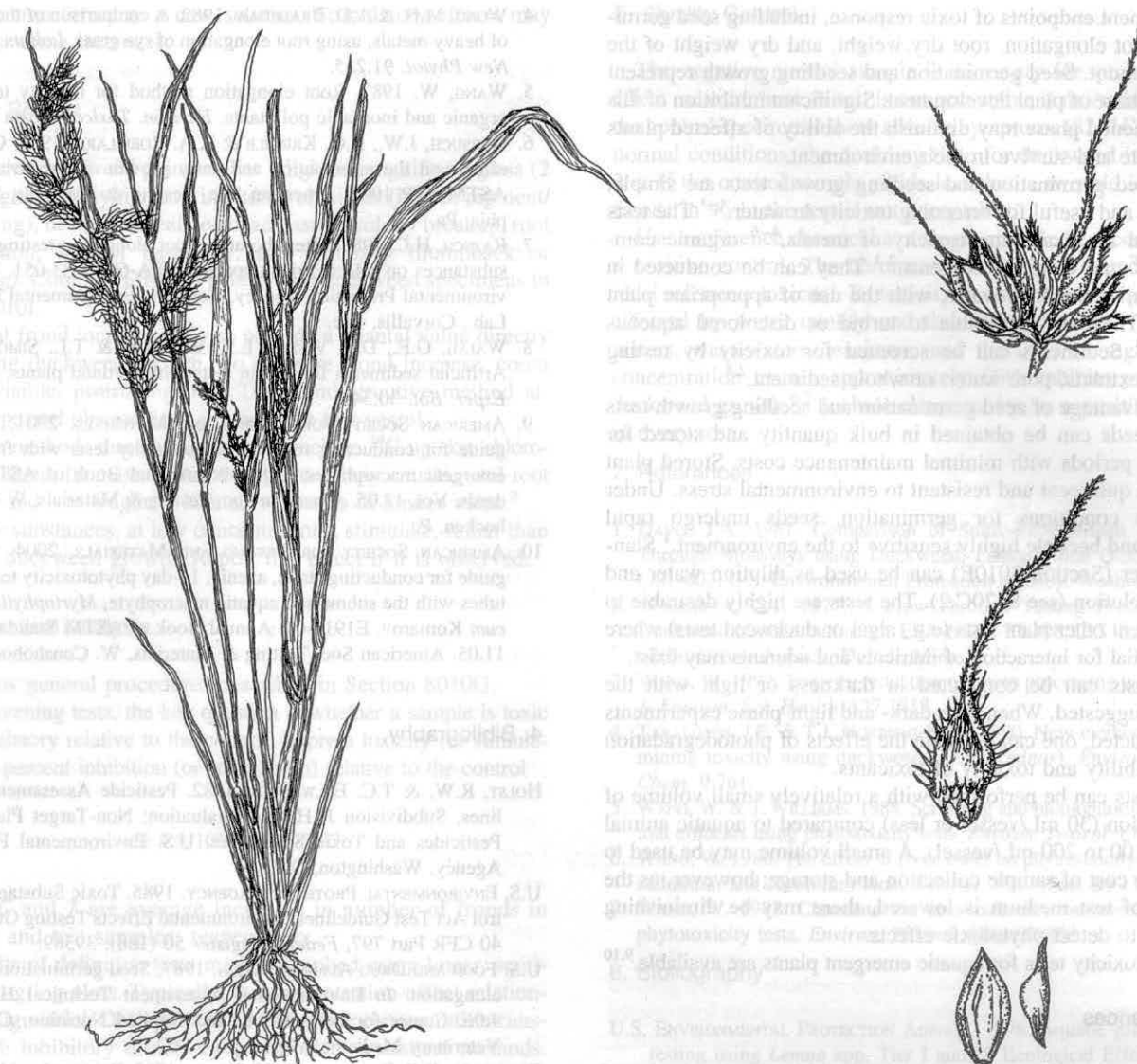


Figure 8220:1. *Echinochloa crusgalli* (Japanese millet or duck millet). Left: entire plant; right, top to bottom: spikelet cluster, spikelet, and floret seed.

Echinochloa crusgalli

(Figure 8220:1)

Leersia oryzoides

Nelumbo lutea

Oryza sativa

Rorippa nasturtium-aquaticum

Zizania aquatica

Japanese (duck)

millet

Rice cutgrass

American lotus

Rice

Watercress

Wild rice

The germination of American lotus seeds requires scarification that may affect toxicity test results.⁵

Obtain species variety (cultivar) and any certification information, such as germination percentage and date collected. Field-collected seeds can be used. Remove weed seed if present. Generally, avoid use of plant seeds treated with fungicides, repellents, or micronutrients (e.g., boron, manganese), etc. If it is desired to use such seeds, ensure that bias does not result.

2. Preparing Test Organisms

Obtain sufficient seeds for one year of testing. Store fresh seeds at -10°C (or 4°C if seeds cannot tolerate lower temperature). Test seeds regularly during storage for percent germination. Some species can be stored longer than one year without a decrease in percent germination.

Use seeds from the same storage lot and year or season of collection. Before beginning toxicity testing, separate seeds into size classes using standard seed dockage sieves. Discard damaged seeds. Use the size class that gives the highest, uniform germination rate exclusively for the test.

3. References

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8220 C. Toxicity Test Procedure

1. General Considerations

Seed germination and seedling growth tests may be conducted using either static, renewal, or flow-through methods. As a general rule, if test samples are not highly volatile or degradable, use a static test. Otherwise, use a renewal (daily renewal) or flow-through method with fresh sample (see Section 8010D).

2. Preparing Test Materials

Use reconstituted fresh water, Table 8010:I, as dilution water and control water. For experiments limited to seed germination and initial root growth, use deionized or distilled water with amendments for the control and add the pollutant to test samples. For growth studies, use the culture medium, Table 8010:IV.A and B, and buffer to desired pH levels (hardness also can be adjusted) for the controls and the test media.

For preparation of toxicant solutions, see Section 8010F.2b.

3. Test Procedures and Conditions

Use these procedures in screening, range-finding, or definitive tests.

In a screening test, use a predetermined concentration (e.g., 100% effluent) to determine if a sample is inhibitory in comparison with the control. If a sample is inhibitory by more than 10%, test it further by using range-finding and definitive tests. In the range-finding test, examine at least three concentrations, usually at ratios of 0.1, e.g., 10%, 1%, and 0.1%, plus the control water.

Devise a definitive test on the basis of range-finding test results. Use at least five concentrations of toxicant solutions in a ratio of 0.5, e.g., 10%, 5%, 2.5%, etc. Ideally, prepare a series of solutions in which the midpoint concentration produces an inhibitory effect of approximately 50% and the highest and lowest concentrations produce approximately 90 and 10% inhibition. Stimulatory effects may occur in some instances.

Use four replicates of each test treatment and control. Include control water in each test. Use a 100- × 15-mm or 47-mm culture dish, test tube, or equivalent, as the test vessel. Use only one kind of vessel through each test. Do not use filter paper or seed pack growth pouch, because it may cause erratic results.¹⁻³ Pipet 8 or 10 mL test solution into each test vessel. Seed trays may be used, requiring 30 mL test solution.³

Place 10 to 15 seeds in each test vessel. Seeds should not contact each other or the sides of culture dishes. Place test vessels in a seed germinator or other growth facility. Use a randomized complete block design with blocks delineated within

TABLE 8220:I. EXAMPLE OF SEED GERMINATION AND SEEDLING GROWTH TEST CONDITIONS

Test Variable	Condition or Value in Example Test
Test species	<i>Echinochloa crusgalli</i> (Japanese millet)
Pretreatment	20 min, hypochlorite solution (3.33 g OCl ⁻ /L)
Test type	Static or renewal
Temperature	25 ± 1°C
Light quality	Dark or light
Test vessel	100- × 15-mm culture dish
Test solution	8 mL/vessel
Specimens	15 seeds/vessel
Replicates	4
Control solution and dilution water	Standard water
Test duration	120 h
Indicators	Seed germination (radicle 5 mm or longer) Root elongation Root dry biomass Shoot dry biomass Abnormal appearance

the growth facility; if blocking is not feasible, totally randomize vessels in the growth facility.

Test duration, temperature, and light regimes will depend on experimental design and test species. Typically, incubate seeds until the expected percentage of control seed germination has been attained (§ 5 below) and control seed root elongation reaches at least 20 mm.⁴

Table 8220:I provides an example of appropriate test conditions.

If fungi or other microorganisms interfere with germination, pretreat test seeds with a reagent-grade sodium hypochlorite solution (3.33 g OCl⁻/L) or 10% household bleach for 20 min. Rinse pretreated seeds eight to ten times with deionized or distilled water. Remove excess solution with tissue paper. Omit pretreatment step if this affects germination of the control by more than 10%.

Conduct the renewal test, if necessary, by transferring seeds or seedlings into clean vessels containing fresh toxicant or control water daily. Alternatively, modify a proportional diluter for flow-through testing.

4. Test Results

A seed is counted as having germinated if the radicle reaches a length of 5 mm or longer. Record all root and shoot elongation (to 1 mm) of each germinated seed in each dish.

Measure length of each primary root from the transition point of the hypocotyl to the tip of the root. Use a digitizer interfaced with a computer, if available.

Additionally, cut primary roots in each dish, combine, dry to constant weights at 70°C, and weigh. Likewise determine shoot biomass. Report abnormal appearance such as discoloration, stunted growth, and chlorosis.

Some substances, at low concentration, stimulate, rather than inhibit, seed germination or root elongation. Report this effect if it is observed.

5. Statistical Analysis

Follow general procedure described in Section 8010G.

Express sample toxicity in percent inhibition relative to the control.^{5,6}

$$\% I = 100 (C - T)/C$$

where C and T are mean seed germination percentages in control water and test solutions, respectively, if seed germination is used as the test indicator. If root elongation is used as the test indicator, C and T are root length (in mm) in control and test solutions, respectively.

Determine IC₅₀ value (the concentration causing 50% inhibitory effect) by statistical curve-fitting methods. Report concentration-effect relationship and confidence limit of test results.

6. Quality Control

The negative control sample is needed for quality control and effective measurement. Derive empirical performance criteria (e.g., germination percent, mean and standard deviation of root elongation of the negative control sample, and test duration for

primary root reaching 20 mm) and statistical confidence limits for each species for 3 months. Any time the performance of a test in the next 3 months falls below the confidence limits, repeat tests; if two consecutive tests fall below the criterion, replace the seed lot and discard the data. Use a positive control, 10 mg/L Cr (III), in every test as an additional, long-term quality control. Develop statistical confidence limits of the positive control.

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8310 CILIATED PROTOZOA*

8310 A. Introduction

1. General Discussion

Ciliated protozoans (Kingdom Protista, Phylum Ciliophora) are ubiquitous unicellular eukaryotes that inhabit freshwater and marine aquatic environments, soils, and sediments. Ciliates are important organisms in the transfer and transformation of nutrients in ecological food chains.^{1,2} In the aquatic environment, ciliates are an integral part of the zooplankton community, feed predominantly on bacteria and small phytoplankton,³⁻⁵ and mediate the transfer of energy from the microbial food web to larger

metazoan zooplankton.¹ In soils and sediments, ciliates feed primarily on bacteria and organic detritus.⁶ The prevalence of this group and their importance in trophic processes make them particularly appropriate as organisms used for the assessment of water quality.⁷⁻⁹

Recent advances in the assessment of environmental toxicity have focused on microscale testing, more rapid bioassessment techniques, and more sensitive indicators of water quality (i.e., sublethal versus lethal effects).¹⁰ The potential for using ciliates to evaluate water quality was recognized some time ago.^{11,12} More recently, investigators have focused increasingly on ciliates as test and/or indicator organisms for the assessment of eutrophic and contaminated media, because they represent a neglected trophic level in most toxicity test batteries, and are sensitive to a broad range of toxicants in the natural environ-

* Approved by Standard Methods Committee, 1997.

Joint Task Group: 20th Edition — Guy L. Gilron (chair), Sharon G. Berk, James R. Pratt.

ment.¹³ A comprehensive review of this field can be found elsewhere.¹⁴

2. Selection of Method

This section includes three standardized toxicity test methods using ciliated protozoa as test organisms. The first two utilize ciliates common in fresh water, and can be used for whole water testing with effluents and pure chemicals, while the third utilizes a soil ciliate; this test is most appropriate as an elutriate test, where contaminated soils and mine tailings are potentially implicated.

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8310 B. Growth Inhibition Test with Freshwater Ciliate *Colpidium campylum*

1. Background

The short generation time of ciliates and the usefulness of growth as a sensitive biological characteristic have made growth inhibition a widely used end point for toxicity tests. Such tests measure population growth rate of the test ciliate species in response to a gradient of test concentrations.

This method, a short-term toxicity test,¹⁻⁴ is based on a change in the population growth of *Colpidium campylum* (Figure 8310:1) over a 24-h period. The number of cells produced during 24 h in the presence of the toxicant is compared to the growth in a control culture. The toxicity test has a broad range of application for single toxicants and contaminant mixtures such as effluents. Intercalibration data and technical review⁵ support its usefulness as a standard method.

Other examples of test methods that have used growth inhibition as a test end point for aquatic assessments are described elsewhere.^{6,7}

2. Source of Test Organisms

Cultures of *Colpidium campylum* (ATCC 50414) can be obtained from the American Type Culture Collection.*

* ATCC, Rockville, MD.

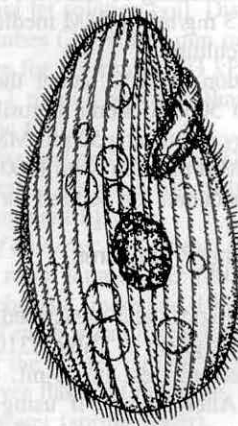


Figure 8310:1. *Colpidium campylum*.

3. Holding and Culturing Test Organisms

a. Culture maintenance: Culture *Colpidium campylum* Stokes axenically in Proteose Peptone Yeast Extract and Serum (PPYS) medium⁸ enriched with bovine serum albumin.^{9†} Incubate cultures at 28°C in the dark; subculture each week.

† A4503, Sigma Chemical Co., St. Louis, MO, or equivalent.

TABLE 8310:I. SUMMARY OF ECOLOGICAL AND TESTING CONDITIONS FOR THE FRESHWATER CILIATE *COLPIDIUM CAMPYLOM*

Condition	Description
Geographical distribution	Cosmopolitan
Habitat	Littoral and benthic zone of ponds and lakes
Length of life cycle	Cell cycle: 2–5 h
Type/duration of test	Growth rate; 24-h EC50
Test temperature	28°C
Light cycle	Regular ambient lighting
Validity criteria	None provided
End points	Growth inhibition, EC50
Reference toxicant	Potassium dichromate

b. Preparing organisms for testing: Acclimate organisms to monoxenic cultivation. Grow them with commercially available lyophilized *Escherichia coli*, strain ATCC 11303,‡ strain ATCC 9637,§ or strain K12.||

Prepare Minimal Medium (MM) used for the test as follows:

CaCl ₂ ·2H ₂ O	107 mg
NaCl	14.5 mg
NaNO ₃	4.5 mg
MgSO ₄ ·7H ₂ O	75.7 mg
Na ₂ SO ₄	39.5 mg
NaHCO ₃	135 mg
Reagent water	1 L

Mix thoroughly. The pH of the medium should be 8.15 ± 0.02. Filter through a 0.45-μm membrane filter and store at 4°C.

Inoculate two 125-mL sterile borosilicate erlenmeyer or sterile cell culture bottles# containing 10 mL MM medium and 0.4 mL *E. coli* suspension (2.5 mg/mL in MM medium) with 2 drops of axenic *C. campylo* culture.

After 48 h incubation at 28°C, count the cells. Prepare the definitive inoculum in 500-mL sterile borosilicate erlenmeyer or cell culture bottles** by inoculating 50 mL MM medium and 2 mL *E. coli* (2.5 mg/mL in MM medium) with 1000 cells/mL. After 48 h growth at 28°C, the inoculum can be used for the toxicity test.

4. Test Conditions and Procedures

A summary of ecological and prescribed test conditions for *Colpidium campylo* is given in Table 8310:I.

a. Test vessels: Perform test in 30-mL crystal polystyrene screw-capped vials. Alternatively, if using electronic particle counting, use counter cuvettes directly. If products tested can adsorb on plastic or alter it, use borosilicate glass or TFE vials.

b. Test initiation: To each vial add in the given order:

Toxicant solution in MM (1.25 final concentration in the vial)	4 mL
<i>E. coli</i> , suspension 2.5 mg/mL (in MM medium)	0.25 mL
<i>C. campylo</i> dilution (3333 cells/mL)	0.75 mL

‡ Sigma EC11303.

§ Sigma EC9637.

|| Sigma EC1.

Nunclon, 50 mL, 25 cm², or equivalent.

** Corning, 270 mL, 75 cm², or equivalent.

Start test timing when the ciliates are added. The final volume is 5 mL with an initial cell concentration of 500 cells/mL. For each test on a substance, use one vial per concentration and three control vials (i.e., without toxicant).

To verify true value of the inoculum (500 cells/mL in theory), distribute 0.75-mL portions of the 3333 cells/mL dilution in three separate vials, add 0.225 mL MM, and fix with 1 mL commercially prepared 2.5% glutaraldehyde solution. Count samples and calculate densities. The mean of the three values is considered as the initial concentration (N_0). Incubate vials in the dark at 28°C for 24 h.

At the same time, initiate a reference toxicant test (with potassium dichromate) with an appropriate range of concentrations to verify the sensitivity of the biological material. An EC50 of 10 to 15 mg potassium dichromate/L indicates acceptable test system quality control.¹

c. Counting and calculation: At end of incubation period, fix each vial with 1 mL commercially prepared 5% glutaraldehyde solution. Count ciliate cells either electronically, with a particle counter fitted with a 200-μm aperture probe, after dilution with a 1% NaCl electrolyte solution filtered through a 0.45-μm membrane filter; or manually, using microscopy and the aid of a counting chamber (such as a hemocytometer, Palmer cell, or Sedgwick-Rafter cell). Compute the number of cells produced (CP) by:

$$CP = N - N_0$$

where:

N = final counted population, and

N_0 = initial concentration, ¶ 1 above.

5. Evaluating and Reporting Test Results

The statistical end point of the test is the EC50. The cells produced in each concentration of toxicant are estimated as percentage of the control (mean of the three vials). Determine the EC50 by using a computer program, e.g., Stephan LC50 program¹⁰; (see Section 8010G).

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8310 C. Chemotactic Test with Freshwater Ciliate *Tetrahymena thermophila*

1. Background

The movement of ciliates toward or away from chemicals (i.e., chemosensory behavior) is a well-studied physiological response in ciliates.^{1,2} There are several tests that measure chemosensory behavior or the inhibition of chemosensory behavior as the biological end point.³⁻⁵

Chemotaxis inhibition toxicity tests have a broad range of application for single toxicants and contaminant mixtures such as effluents. The T-maze toxicologic assay (TMTA)⁶, upon which this method is based, has undergone species comparison validation, technical refinement, and interlaboratory calibration.

2. Source of Test Organisms

Cultures of *Tetrahymena thermophila* (Figure 8310:2) [ATCC 30382 Strain B-18684 (1975) or ATCC 30383 Strain B—18686

(1975)] can be obtained from the American Type Culture Collection.*

3. Holding and Culturing Test Organisms

a. *Culture medium preparation:* Prepare Proteose Peptone Yeast Extract (PPYE) medium as follows (depending on culture medium requirements):

Dextrose	0.5 g
Proteose peptone†	2.0 g
Yeast extract†	2.0 g
Distilled water	400.0 mL

Heat distilled water in a beaker over a Bunsen burner. Add dextrose and stir. Add proteose peptone and mix. Add yeast extract, but *do not* stir. Heat solution until yeast extract is dissolved, but do not let solution boil. Dispense 10-mL portions into culture (test) tubes (20 × 150 mm or 15 × 150 mm). Cap tubes and autoclave for 20 min at 103 kPa. The shelf life of the culture medium is 1 month, provided that it is refrigerated and covered with plastic film.‡

b. *Culture transfer and maintenance:* Using sterile technique, transfer culture every 2 weeks. Keep cultures at room temperature with regular ambient lighting.

c. *Preparation of cultures:* Ensure that all solutions used in the toxicity test are at room temperature (20° ± 2°C).

Using sterile technique, inoculate 10 mL sterile PPYE with about 1 mL stock *Tetrahymena thermophila* culture. After 48 h, aseptically transfer the 10-mL culture to 50 mL sterile PPYE in a 250-mL erlenmeyer flask. At this time, soak the corks for the mazes in dilution water (spring water).

After 24 h, harvest 50 mL PPYE culture by centrifuging in centrifuge tubes (preferably use conical 12- to 15-mL tubes) at 1200 rpm for 3 min. With a Pasteur pipet, remove the supernatant, ensuring that the pellet of cells is not disturbed.

Gently and completely resuspend the pellets into a centrifuge tube and add 12 to 15 mL dilution water. Centrifuge at 1200 rpm for 3 min. Repeat resuspension, centrifugation, and dilution twice more, removing supernatant each time. Gently and completely resuspend the pellet in a small amount of dilution water.

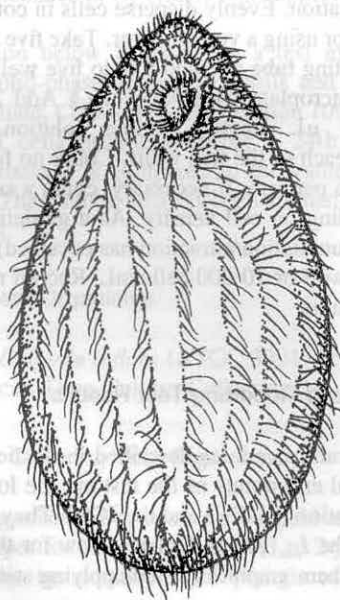


Figure 8310:2. *Tetrahymena thermophila*.

* ATCC, Rockville, MD.

† Difco or equivalent.

‡ Parafilm™ or equivalent.

TABLE 8310:II. SUMMARY OF ECOLOGICAL AND TEST CONDITIONS FOR THE FRESHWATER CILIATE *TETRAHYMENA THERMOPHILA*

Condition	Description
Geographical distribution	Cosmopolitan
Habitat	Littoral and benthic zone of ponds and lakes
Length of life cycle	Cell cycle: 2–5 h
Type/duration of test	Chemotaxis; 20-min IC50
Test temperature	20°C
Light cycle	Regular ambient lighting
Validity criteria	Test is invalid if mean control I_{tox} value is outside the range 0.43–0.57; total cell counts for each replicate are ≥ 200 .
End points	Chemotaxis, LOEC, IC50
Reference toxicant	Sodium chloride

Transfer cells with a Pasteur pipet to 50 mL dilution water in a 250-mL erlenmeyer flask. Leave for 18 h under conditions of ambient temperature and lighting conditions to starve the culture.

Harvest cells by centrifuging at 1200 rpm for 3 min and carefully removing supernatant with Pasteur pipet. Gently and completely resuspend the pellets into one centrifuge tube. Using dilution water, adjust cell density to approximately 400 000 cells/mL ($\pm 10\%$).

4. Test Conditions and Procedures

A summary of ecological and prescribed test conditions for *Tetrahymena thermophila* is given in Table 8310:II.

a. Test apparatus and design: A schematic diagram outlining the apparatus used in the TMTA procedure is presented in Figure 8310:3. The test design comprises at least three replicate glass T-mazes, 30 cm in longest dimension, for each concentration, with five concentrations in a serial dilution and a control. Before running the definitive test, perform a preliminary motility test to ensure that cells are motile in the test medium. For a full test, set up five concentrations and a control, each comprising three replicate T-mazes at each concentration (total of 18 mazes).

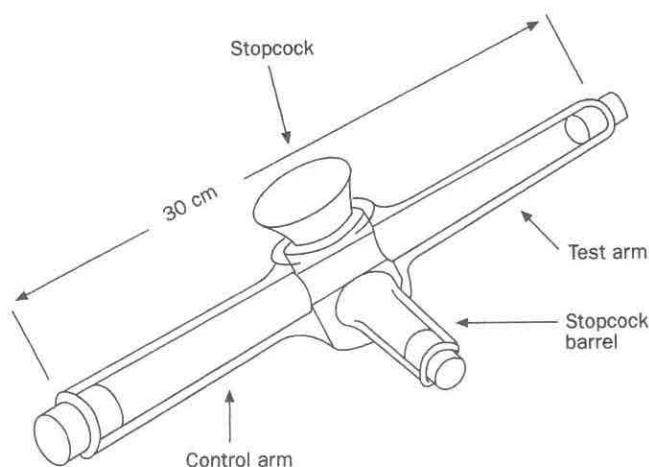


Figure 8310:3. Test apparatus for T-maze chemotactic test.

b. Test exposure: Turn stopcock for each maze so that the bore is in line with the third (upright) arm. Label maze arms "test" and "control" with tape and/or marker. Using Pasteur pipets (14.6-cm/5.75-in.), fill arms of each T-maze apparatus, one at a time, with the respective solutions [one test (toxicant solution), one control, in that order]. Stop each arm with a rubber cork. Ensure that no air bubbles are caught in the arms, particularly around the stopcocks. Holding one arm upwards at a 45-deg angle, shake out all air bubbles by firmly hitting the T-maze apparatus on the palm of the hand; repeat for second arm. Recork, if necessary, to release any air bubbles.

Using a 23-cm Pasteur pipet, transfer the cells from a homogeneous suspension into each stopcock barrel (the solution is filled above the level of the bore). Tap bottom of each T-maze apparatus gently to remove any initial air bubbles. Remove air bubbles from all stopcock barrels before commencing the test for all mazes. NOTE: Removal of air bubbles is crucial to conducting the assay properly, because bubbles will prevent organisms from migrating into the arms.

After all T-mazes are filled completely, begin 20-min exposure period by turning the stopcock so that cells are able to migrate freely through the arms. Grease stopcocks sparingly with high-vacuum grease before use. Ensure that the stopcock barrel is completely aligned with the stopcock arms. After 20-min exposure period, turn stopcocks again to a closed position to terminate the test.

In parallel with each run of the T-maze tests, perform a standard reference toxicant test (using sodium chloride) with an appropriate range of concentrations to verify the sensitivity of the biological material. A lowest-observed-effect concentration (LOEC) of 2000 to 3000 mg/L indicates acceptable test system quality control.⁶

c. Test termination and enumeration: Immediately after the test is completed, empty arms of T-maze into counting tubes (e.g., test tubes, Coulter counter cuvettes). Using a 14.6-cm Pasteur pipet, rinse each arm with the test solution from that arm to ensure that all cells have been removed. Enumerate cells under 400 $\times$ magnification. Evenly disperse cells in counting tubes by inverting tubes or using a vortex mixer. Take five 10- μ L samples from each counting tube and add this to five wells of a polystyrene 96-well microplate with flat wells. Add 20 μ L dilution water and 10 μ L Lugol's iodine solution (see Section 10200B.2a) to each of the five wells. Count no fewer than three of the five wells per arm. If necessary, count a smaller or larger portion, depending on cell density. As a guideline, the densest arm (where accumulation/attraction has occurred) should have at least 100 cells/well or 10 000 cells/mL. Record replicate counts and average results.

5. Evaluating and Reporting Test Results

Follow general procedures described in Section 8010G.

The statistical end points of the test are the lowest-observed-effect concentration (LOEC) and the IC50. They are determined by calculating the I_{tox} values defined below for the concentration series, plotting them graphically, and applying statistical analysis.

⁶ Corning No. 25880-96 or equivalent.

Calculate a "toxicity" index (I_{tox}) for each T-maze as follows:

$$I_{tox} = \frac{T}{T + C}$$

where:

T = mean number of cells in test arm, and

C = mean number of cells in control arm.

To determine LOEC value, plot I_{tox} values for concentrations tested and a control (y-axis) against concentration of the toxicant (x-axis). When there is a response, an increase in I_{tox} with increasing concentration denotes attraction, and a decrease in I_{tox} with increasing concentration denotes repulsion.

Conduct an analysis of variance (ANOVA) and a multivariate test (e.g., William's or Dunnett's tests)⁷ on all data for a given test, to determine the lowest concentration at which the I_{tox} value is statistically, significantly different from the control I_{tox} . That concentration is the LOEC.

Calculate the IC50 by the linear interpolation method⁸ with a software package (e.g., ICPIN).||

|| BOOTSTRP, available from U.S. Environmental Protection Agency, Cincinnati, Ohio.

8310 D. Growth Inhibition Test with the Soil Ciliate *Colpoda inflata*

1. Background

This toxicity test measures the population growth rate of the test ciliate species in response to a gradient of test concentrations. It is similar to that used for algal growth inhibition tests.^{1,2} Growth, in this case, may be inhibited by direct effects on the ciliate cell maintenance or by effects that suppress energy intake (i.e., feeding).

The description below is based on a test method³ for the evaluation of solid-phase media (i.e., soils and soil elutriates) using the soil ciliate, *Colpoda inflata* (Section 10900, Plate 6:D). The number of cells produced during a 24-h period in the presence of a toxicant is compared to the value obtained in a control culture. The method also has been applied successfully to mining effluents.⁴

2. Source of Test Organisms

Dry cysts of *Colpoda inflata* (ATCC 30917) can be obtained from the American Type Culture Collection.*

3. Holding and Culturing Test Organisms

a. Holding organisms: Dry cysts can be held at room temperature on filter paper for extended periods (1 to 2 years). Cysts in spent cultures can be stored wet for periods of up to months

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without loss of viability. Grow these cultures or dry cysts as needed by adding culture medium as described below.

b. Maintaining cultures: Maintain cultures developed from stored cysts for 2 to 7 d in 10% Sonneborn's *Paramecium* medium, prepared as follows: Boil 2.5 g cereal grass leaves† for 5 min in 1 L distilled, deionized water; filter,‡ adjust volume to 1 L with distilled water, and add 0.5 g Na₂HPO₄. Dilute full-strength medium before use with distilled, deionized water and autoclave in 50-mL portions. Add to cultures a food bacterium such as nonpathogenic *Klebsiella pneumoniae* (ATCC 27889) as recommended by ATCC.

4. Test Conditions and Procedures

A summary of ecological and prescribed test conditions for *Colpoda inflata* is given in Table 8310:III.

a. Test vessels: Conduct tests in sterile, 24-well, polystyrene tissue culture plates,§ using either 10% Sonneborn's medium (§ 3b above) or a minimal salts medium consisting of 6 mg KCl, 4 mg CaHPO₄, and 2 mg MgSO₄ in 1 L sterile distilled, deionized water. Test volume is 2 mL per well.

b. Test initiation: Dispense sterile medium into wells and amend with toxicant from stock solutions of reagent-grade chemicals prepared in sterile distilled, deionized water. Alternatively, conduct tests using percentage dilutions of complex mix-

† Cerophyl®, Agri-Tech, Inc., Kansas City, MO 64112, or equivalent.

‡ Whatman No. 1 filter paper or equivalent.

§ Costar, Inc. or equivalent.

* ATCC, Rockville, MD.

TABLE 8310:III. SUMMARY OF ECOLOGICAL AND TEST CONDITIONS FOR THE SOIL CILIATE *COLPODA INFLATA*

Condition	Description
Geographical distribution	Cosmopolitan
Habitat	Soil, vegetation litter
Length of life cycle	Cell cycle: 2–5 h
Type/duration of test	Growth; 24-h IG50
Test temperature	Regular ambient lighting
Light cycle	None provided
Validity criteria	IG50 25–70 $\mu\text{g/L}$ for reference toxicant test
End points	Growth inhibition, IG50
Reference toxicant	Copper sulfate

tures (e.g., wastewater effluents, soil extracts⁴). For mixture tests, prepare minimal salts medium in concentrated form (10 $\times$) and dilute with sterile distilled water.

After medium and toxicant are dispensed into test wells, add ciliates from log-phase cultures (48 to 96 h old) along with prey bacteria. The volume of well-mixed culture added should ensure that equal numbers of ciliates (approximately 100 cells/mL) are added to each well.

At the same time, perform a reference toxicant test (using copper sulfate) with an appropriate range of concentrations to verify the sensitivity of the biological material. A toxicant concentration corresponding to a 50% inhibition of growth relative to controls (IG50) between 25 and 70 $\mu\text{g/L}$ indicates acceptable test system quality control.

c. Counting and calculation: After 24 h, remove subsamples from each test well and enumerate using a direct counting technique as follows: Thoroughly mix each well with a micropipettor and transfer a 20- μL subsample to a clean microscope slide as 3 or 4 drops. Scan all drops immediately at low magni-

fication (40 $\times$) on a stereomicroscope to search for active cells. Active cells are always moving and can be distinguished easily from bacterial aggregates or cysts. For a given well, repeat subsampling at least three times to assure that an accurate estimate of the population in the well is obtained. If repeat counts of subsamples vary by more than 30%, continue subsampling until population estimates stabilize. Repeat this procedure for each well, and compute the mean of subsample estimates for a given replicate. Alternatively, use electronic particle counting techniques (i.e., Coulter counter) to enumerate cells.

5. Evaluating and Reporting Test Results

Follow general procedures described in Section 8010G.

Estimate IG50 by regressing cell number on log dose and then using inverse prediction⁵ to estimate IG50 from the control response (mean = 100%).

6. References

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8420 ROTIFERS*

8420 A. Introduction

1. Ecological Significance

Rotifers are classified in the Phylum Rotifera, one of several phyla of lower invertebrates. There are approximately 2000 rotifer species named; they are divided into two classes, Digononta and Monogononta.^{1,2} Monogononts reproduce parthenogenetically, but in response to specific environmental cues they reproduce sexually, yielding dormant embryos called cysts (resting eggs), which have been used in toxicity testing.³ The use

of rotifer cysts for toxicity testing has been discussed in the literature.³ Most rotifer species inhabit fresh water,⁴ but there are some genera, like *Synchaeta*, in which most species are marine.⁵ In coastal marine habitats, rotifers sometimes are the dominant portion of the biomass.⁶ They also are abundant in marine interstitial habitats, interstitial water of soils,⁷ and water clinging to mosses, liverworts, and lichens.⁸ In freshwater lake plankton⁹ and in river sediments,¹⁰ rotifers often are abundant, with high species diversity.

Rotifers play an important role in the ecological processes of many aquatic communities.¹¹ As suspension feeders, planktonic rotifers influence algal species composition through selective grazing.^{12–14} Rotifers often compete with cladocera and cope-

* Approved by Standard Methods Committee, 1997.
Joint Task Group: 20th Edition — Terry W. Snell (chair), David B. Dusenbery, Colin R. Janssen, Michael C. Newman, Guido Persoone.

pods for phytoplankton in the 2- to 18- μ m size range. Along with crustaceans, rotifers contribute substantially to nutrient recycling.¹⁵ Rotifers are food for many fish larvae.¹⁶

2. Types of Toxicity Tests

The procedures in Section 8420 serve as guidelines for using rotifers to estimate sublethal toxicity with population growth rate as endpoint. These procedures have been adapted for examining surface water and effluents as well as sediment pore water. Several other types of rotifer tests described are based on endpoints such as mortality,¹⁷ ingestion,¹⁸ swimming,¹⁹ enzyme activity,²⁰ and stress protein gene expression.²¹

3. References

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8420 B. Selecting and Preparing Testing Organisms

1. Selecting Test Organisms

The rotifers recommended for use were chosen because of the existence of published reports describing protocols, a database of responses to pure toxicants, and the availability of cysts. In *Brachionus calyciflorus* and *B. plicatilis*, for which standardized tests exist, the recommended strain also is indicated. A summary of ecological and test conditions to be considered in tests with these organisms is given in Table 8420:I. Substantial differences in sensitivity to toxicants have been reported among rotifer strains of different geographic origin.¹ In accord with the criteria listed in Section 8010E.1, the recommended test species include (but are not restricted to) the following:

a. Freshwater rotifers:

Class: Monogononta

Brachionus calyciflorus (Gainesville strain)²

*Brachionus rubens*³

*Brachionus patulus*⁴

*Asplanchna brightwelli*⁵

Class: Digononta

*Philodina roseola*⁶

*Philodina acuticornis*⁷

See Section 10900, Plate 8 for drawings of several freshwater rotifer species.

b. Marine rotifers:

Brachionus plicatilis (Russian strain)¹

2. Obtaining Test Organisms

a. Rotifer cysts: *B. calyciflorus* in fresh water and *B. plicatilis* in marine waters are hatched from cysts. Rotifer cysts hatch synchronously, providing test animals of similar age in uniform physiological condition. Detailed descriptions of rotifer cyst hatching are available.^{1,2}

b. Cyst hatching: To initiate hatching, place about 40 mL standard fresh water in a glass petri dish or tissue-culture-grade polystyrene dish. Incubate rotifer cysts at 25°C in light of 3000 to 4000 lux. Hatching should start after about 15 to 16 h; within 2 h remove dish from incubator to transfer rotifers to test tubes. Cooler temperatures, low or high pH, and elevated hardness and

TABLE 8420-I. SUMMARY OF ECOLOGICAL AND TEST CONDITIONS THAT SHOULD BE CONSIDERED WHEN CONDUCTING TOXICITY TESTS WITH *B. CALYCIFLORUS* (BC) OR *B. PLICATILIS* (BP) ROTIFERS

Condition	Comment
Geographical	Pan-global distribution
Habitat	Pelagic zooplankton
Life cycle	Parthenogenetic and sexual reproduction
Lifespan	5–7 d at 25°C
Temperature	10–32°C
Salinity	BC – 0–5, BP – 1–60
Nutrition	Suspension feeders on microalgae
Photoperiod	No special requirements
Control mortality	Not to exceed 10%
Reproductive test	Water, pore water ¹

alkalinity can delay hatching. If hatching is delayed, check cysts hourly to ensure collecting test animals within 2 h of hatching.

Hatch *B. plicatilis* cysts by a similar procedure in standard synthetic seawater. *B. plicatilis* cysts usually initiate hatching after 24 to 26 h at 25°C in light of 3000 to 4000 lux.

c. *Food and feeding*: See ¶ C.3 below.

d. *Rotifer reproduction*: Rotifers reproduce asexually via ameiotic parthenogenesis.⁸ Monogononts also can reproduce sexually, but this capacity usually is not utilized in toxicity tests (with certain exceptions⁹). Asexual rotifer reproduction allows simple sublethal toxicity tests to be conducted using population growth as an end point.

3. Parasites and Diseases

Fungal parasites on rotifers have been reported in a few natural populations, but never in laboratory populations used for

toxicity testing. No known diseases affect the use of brachionid rotifers in toxicity tests.

4. References

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8420 C. Aquatic Toxicity Test Procedures

1. General Procedures

Use exploratory tests¹ to determine the toxicant concentrations for short-term tests (see Section 8010D). Prepare control and test solutions in standard synthetic fresh water or seawater and introduce them into test containers as described in Section 8010F.

2. Water Supplies

a. *Artificial fresh water*: See Section 8010E.4b1) and Table 8010:I for preparation of a moderately hard water. Adjust to pH 7.5 with 10M KOH or HCl.

b. *Artificial seawater*: Prepare standard synthetic seawater² with a salinity of 15 by adding 11.31 g NaCl, 0.36 g KCl, 0.54 g CaCl₂, 1.97 g MgCl₂·6H₂O, 2.39 g MgSO₄·7H₂O, and 0.17 g NaHCO₃ to 1 L deionized or distilled water. Mix well on a magnetic stirrer and adjust pH to 8.0 with 10M KOH or HCl.

c. *Deionized water*: Prepare all media with high-quality deionized or distilled water (see Section 1080). Water from certain commercially available systems* is suitable.

3. Food and Feeding

The following procedures are recommended for growth of food organisms. However, other diets, such as a mixture of *Selenastrum capricornutum* and *Chlorella vulgaris* grown on Bold's Basal Medium, have been used successfully.³

a. *Nannochloris oculata* (food for *Brachionus calyciflorus*): Maintain unialgal stock cultures of the green alga *Nannochloris* cells† in sterile test tube cultures containing 20 mL Bold's Basal Medium (BBM), prepared as follows: To 1 L water (¶ 2c), add 250 mg NaNO₃, 75 mg MgSO₄·7H₂O, 175 mg KH₂PO₄, 25 mg CaCl₂·2H₂O, 75 mg K₂HPO₄, 25 mg NaCl, 2 mL vitamin stock (see below), and 2 mL trace metal stock (see below).

* Nanopure II system with one pretreatment, one high-capacity, and two ultrapure cartridges, or equivalent.

† University of Texas at Austin, Culture Collection of Algae, LB 1998.

To prepare 500 mL trace metal stock water, add 2.5 g NaFeEDTA, 11 mg ZnSO₄·7H₂O, 90 mg MnCl₂·4H₂O, 5 mg CoCl₂·6H₂O, 5 mg CuSO₄·5H₂O, and 3.2 mg NaMoO₄·2H₂O to water (§ 2c).

To prepare 500 mL vitamin stock water, add 100 mg thiamine, 5 mg biotin, and 5 mg B₁₂ to water (§ 2c).

Propagate cultures by serial transfer using sterile technique. To inoculate a large *Nannochloris* culture, pour contents of a dark green 20-mL test tube culture into 2 L BBM; this will yield an initial density of about 10³ cells/mL. Aerate this culture with filtered air and maintain at 25°C in light for 3 to 6 d until the cells reach log-phase growth, at which point they have the highest nutritional quality. Harvest and concentrate algal cells by centrifugation at 5000 × g for 10 min. Concentrated algal cells can be stored in the refrigerator for 3 to 4 d without loss of nutritional quality. Quantify algal cell density using a Neubauer slide hemacytometer according to the manufacturer's protocol. Add smallest volume of algae stock necessary to each test solution to make a suspension of 10⁶ cells/mL. Pour 100 mL of each test solution into a 150-mL beaker and stir gently on a magnetic stirrer (approximately 120 rpm) using a small stir bar (about 1.5 cm). Use test solutions promptly; do not stir for more than 30 min. Pipet 12 mL control solution into each of seven replicate test tubes. Repeat for each test solution.

b. Nannochloropsis sp. (food for *Brachionus plicatilis*): Maintain unialgal stock cultures of the Eustigmatophyceae alga *Nannochloropsis* cells in test tubes containing 20 mL of sterile ASPM algal growth medium prepared as follows: To 1 L synthetic seawater (§ 2b), add 150 mg NaNO₃, 10 mg K₂HPO₄, 2 mL trace metal stock (§ 3a), and 2 mL vitamin stock (§ 3a). Propagate cultures by serial transfer using sterile technique. Follow procedures given in § 3a for culturing, concentrating, and dispensing to test tubes.

4. Exposure Chambers

Use standard, disposable 16- × 150-mm borosilicate glass test tubes as exposure chambers.

5. Conducting the Test

An overview of the test is shown in Figure 8420:1.

a. Adding test animals: To begin test, transfer six newly hatched rotifers (neonates) into each test tube. *B. calyciflorus* rotifers are approximately 250 μm in length, about 1/4 the size of newborn *Daphnia*. Their small size and slow swimming speed have some advantages for capturing and manipulation. Newly hatched rotifers are white, so they are most visible on a dark background at about 10 × magnification. The best type of illumination is a darkfield setting. Because they are moderately phototactic, rotifers tend to congregate around the edges of the hatching dish. Squeeze the transfer micropipet gently to provide the right amount of suction. Practice to develop a feel for the right pressure. Confirm that each tube receives exactly six rotifers by watching their entry into the tube under the microscope. Cap and immediately place tubes on a wheel rotator in a 25°C incubator in darkness. Rotation rate should be 10 to 120 revo-

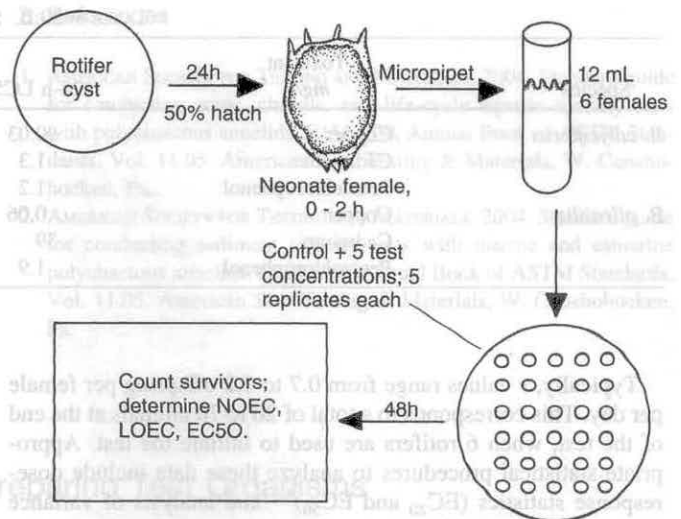


Figure 8420:1. Schematic diagram of rotifer static life-cycle toxicity tests. Test conditions: Generations = 2; end point — reproductive rate $r = (\ln N_t - \ln N_o)/T$; temperature — 25°C; photoperiod — darkness; medium — synthetic freshwater; food — *Nannochloris*.

lutions per hour to maintain the algae in suspension. Do not use a shaker because it will damage the animals. Repeat until all remaining test solutions have been inoculated with rotifers. Record time at which neonates are placed in control treatment as the beginning of the 48-h incubation period.

b. Duration and type of test: The test uses rotifer asexual reproduction to estimate sublethal toxicity. A typical schedule of reproduction is presented in Figure 8420:2. The 48-h population growth rate is calculated and its decline with increasing toxicity quantified.

c. Scoring the test: Remove test tubes from rotator after 48 h. Empty contents of one tube into a petri dish and count number of animals per tube, discriminating between live and dead individuals. Repeat until all tubes have been counted. Calculate r , the population growth rate for each tube as:

$$r = \frac{\ln N_t - \ln N_o}{T}$$

where:

N_t = number of live rotifers in tube after 2 d,
 N_o = initial number of rotifers in tube (6), and
 T = incubation period (2 d).

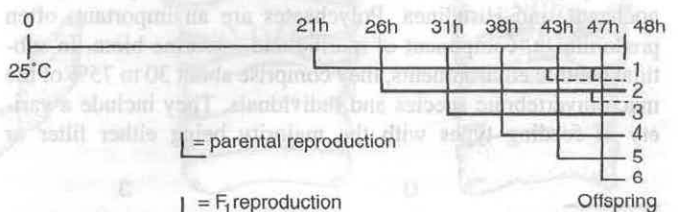


Figure 8420:2. Schedule of reproduction.

TABLE 8420:II. SAMPLE TEST RESULTS

Species	Toxicant mg/L	24-h LC50	48-h Population Growth Rate NOEC	EC50	CV %
<i>B. calyciflorus</i>	Copper	90.03	0.02	0.03	44
	Cadmium	1.3	0.04	0.07	2
	Pentachlorophenol	1.2	0.11	0.27	29
<i>B. plicatilis</i>	Copper	0.06	0.01	—	—
	Cadmium	39	1.0	—	—
	Pentachlorophenol	1.9	0.5	—	—

Typically, r values range from 0.7 to 1.2 offspring per female per day. This corresponds to a total of 20 to 70 animals at the end of the test, when 6 rotifers are used to initiate the test. Appropriate statistical procedures to analyze these data include dose-response statistics (EC_{20} and EC_{50})^{4,5} and analysis of variance followed by a multiple-comparison test, e.g., Dunnett's test, no-observed-effect concentration (NOEC), and lowest-observed-effect concentration (LOEC), (see Table 8420:II).

d. Reference toxicant test: Perform a reference toxicant test, or positive control, with every fifth test. This verifies that the animals will respond to toxicity if it is present. Perform reference tests according to the protocol described above. Cadmium chloride, expressed as cadmium, is commonly used as a reference toxicant. Other metal chlorides or organic compounds may be used.

6. Data Evaluation

The data obtained from this test can be considered valid if the r value in the control is at least 0.70. This represents the minimum acceptable population growth rate. Lower values suggest that there is an unidentified problem with the dilution water, algae, or rotifers. Often when low population growth rates are observed, there is a problem with algae quality. For additional

guidance in data analysis and other statistical considerations, see Sections 8010G and H.

7. References

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8510 ANNELIDS*

8510 A. Introduction

1. Characteristics and Ecology

The phylum Annelida includes three classes: Polychaeta, Oligochaeta, and Hirudinea. Polychaetes are an important, often predominant, component of marine and estuarine biota. In subtidal benthic environments, they comprise about 30 to 75% of the macroinvertebrate species and individuals. They include a variety of feeding types with the majority being either filter or

detritus feeders. Deposit-feeding polychaetes affect surface sediments by their burrowing and irrigating habits. They are important food for snails, large crustaceans, fish, and birds. Many species have short life cycles.

Oligochaetes are among the most common benthic invertebrates in all types of aquatic environments. Particular species assemblages are recognized indicators of environmental quality. In grossly polluted freshwater habitats, oligochaetes dominate the benthic fauna, whereas in estuarine areas they and polychaete worms are often the most common benthic organisms. They feed mainly on bacteria, although other feeding types occur. They affect surface sediments as do the polychaetes. They are an

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Joint Task Group: Donald J. Reish and Peter M. Chapman (co-chairs), Chris G. Ingersoll, David W. Moore, Carol E. Pesch.

important primary or alternate food for leeches, crustaceans, fish, and birds.

Hirudinea are leeches, either free-living or parasitic; they have not been used for toxicity tests.

2. Test Applications

The following procedures are intended to serve as guidelines for the use of polychaetes and oligochaetes in various toxicity tests. These procedures also can be, and have been, adapted to testing sediments.

Other toxicity tests for annelids have been published.^{1,2}

3. References

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8510 B. Selecting and Preparing Test Organisms

1. Selecting Test Organisms

In accord with the criteria listed in Section 8010E.1, the recommended test species include (but are not restricted to) the following:

a. Marine polychaetes:

1) Family Nereidae

Neanthes arenaceodentata (*Neanthes acuminata* and *Neanthes caudata* of some authors) (New England, Florida, California coasts, Europe) (Figure 8510:1A).

Neanthes succinea (all of U.S. coasts).

Neanthes virens (east coast of U.S.).

2) Family Capitellidae

Capitella capitata (cosmopolitan) (Figures 8510:1C and D).

3) Family Ctenodrilidae

Ctenodrilus serratus (cosmopolitan) (Figure 8510:1B).

4) Family Dinophilidae

Dinophilus gyrotilatus (cosmopolitan) (Figure 8510:2A).

5) Family Dorvilleidae

Ophryotrocha diadema (west coast of U.S.) (Figure 8510:2B).

- 6) Other species used in toxicity tests but not discussed include: *Nereis diversicolor*, *Arenicola cristata*, *Abarenicola pacifica*, *Armandia brevis*, and *Polydora cornuta*

b. Freshwater oligochaetes:

1) Family Tubificidae

Limnodrilus hoffmeisteri (cosmopolitan).

Tubifex tubifex (cosmopolitan) (Figure 8510:3A).

Branchiura sowerbyi (cosmopolitan) (Figure 8510:3B).

2) Family Lumbriculidae

Styodrilus heringianus (holarctic) (Figure 8510:3C).

Lumbriculus variegatus (holarctic).

c. Marine oligochaetes:

1) Family Tubificidae

Monopylephorus cuticulatus (N.E. Pacific).

Tubificoides "fraseri" (all North American coasts).

Tectichilus verrucosus (all North American coasts).

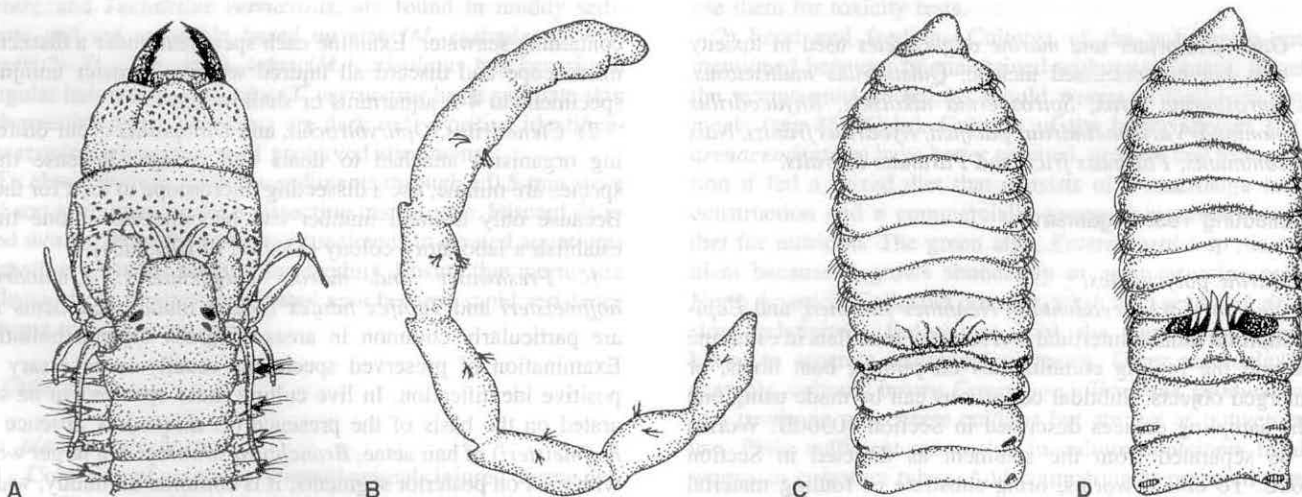


Figure 8510:1. Marine polychaetes. A—*Neanthes arenaceodentata*, anterior end, dorsal view; B—*Ctenodrilus serratus*, adult; C—*Capitella capitata*, female, dorsal view; D—*Capitella capitata*, male, dorsal view.

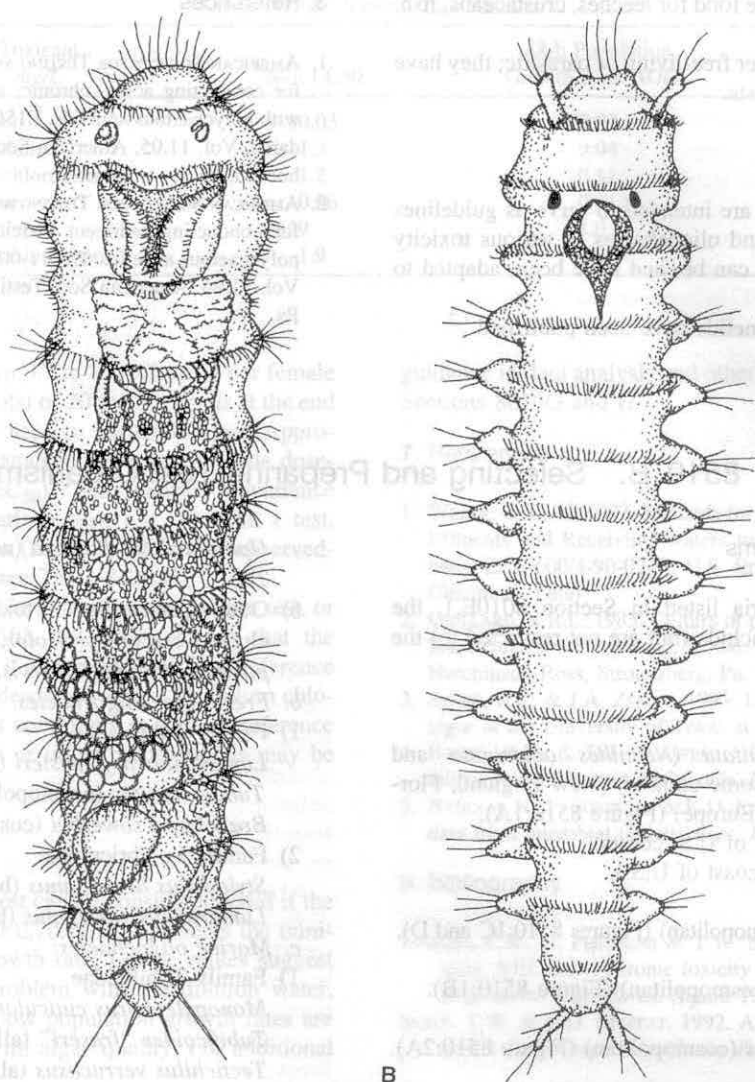


Figure 8510:2. Marine polychaetes. A—*Dinophilus gyrotilatus*, adult; B—*Ophryotrocha diadema*, adult.

- d. Other freshwater and marine oligochaetes used in toxicity tests but not discussed include: *Quistadrilus multisetosus*, *Spirosperma ferox*, *Spirosperma nikolskyi*, *Rhyacodrilus montana*, *Varichaetadrilus pacifica*, *Ilyodrilus frantzi*, *Nais communis*, *Paranais frici*, and *Paranais litoralis*.

2. Collecting Test Organisms

a. Marine polychaetes:

1) *Neanthes arenaceodentata*, *Neanthes succinea*, and *Capitella capitata* inhabit intertidal and subtidal mud flats in estuarine areas and the fouling communities on pilings, boat floats, or submerged objects. Subtidal collections can be made using one of the sampling devices described in Section 10500B. Worms can be separated from the sediment as directed in Section 10500C. To obtain worms, bring substrate or fouling material into the laboratory, place in white enameled pans, and cover with seawater. After a period of time, the worms come to the surface; remove them with a fine brush and transfer to petri dishes

containing seawater. Examine each specimen under a dissecting microscope and discard all injured worms. Transfer uninjured specimens to 4-L aquariums or shallow trays.

2) *Ctenodrilus*, *Ophryotrocha*, and *Dinophilus* occur on fouling organisms attached to floats and pilings. Because these species are minute, use a dissecting microscope to look for them. Because only a small number can be collected at one time, establish a laboratory colony [Section 8510B.3a3)].

b. Freshwater and marine oligochaetes: *Limnodrilus hoffmeisteri* and *Tubifex tubifex* inhabit muddy sediments and are particularly common in areas of gross organic pollution. Examination of preserved specimens usually is necessary for positive identification. In live culture these species can be separated on the basis of the presence (*T. tubifex*) or absence (*L. hoffmeisteri*) of hair setae. *Branchiura sowerbyi* is a larger worm with gills on posterior segments; it is common in muddy, warm-water areas. *Stylodrilus heringianus* is common in areas of clean, fine sand and is identified by the presence of extra rings and nonretractable penes.

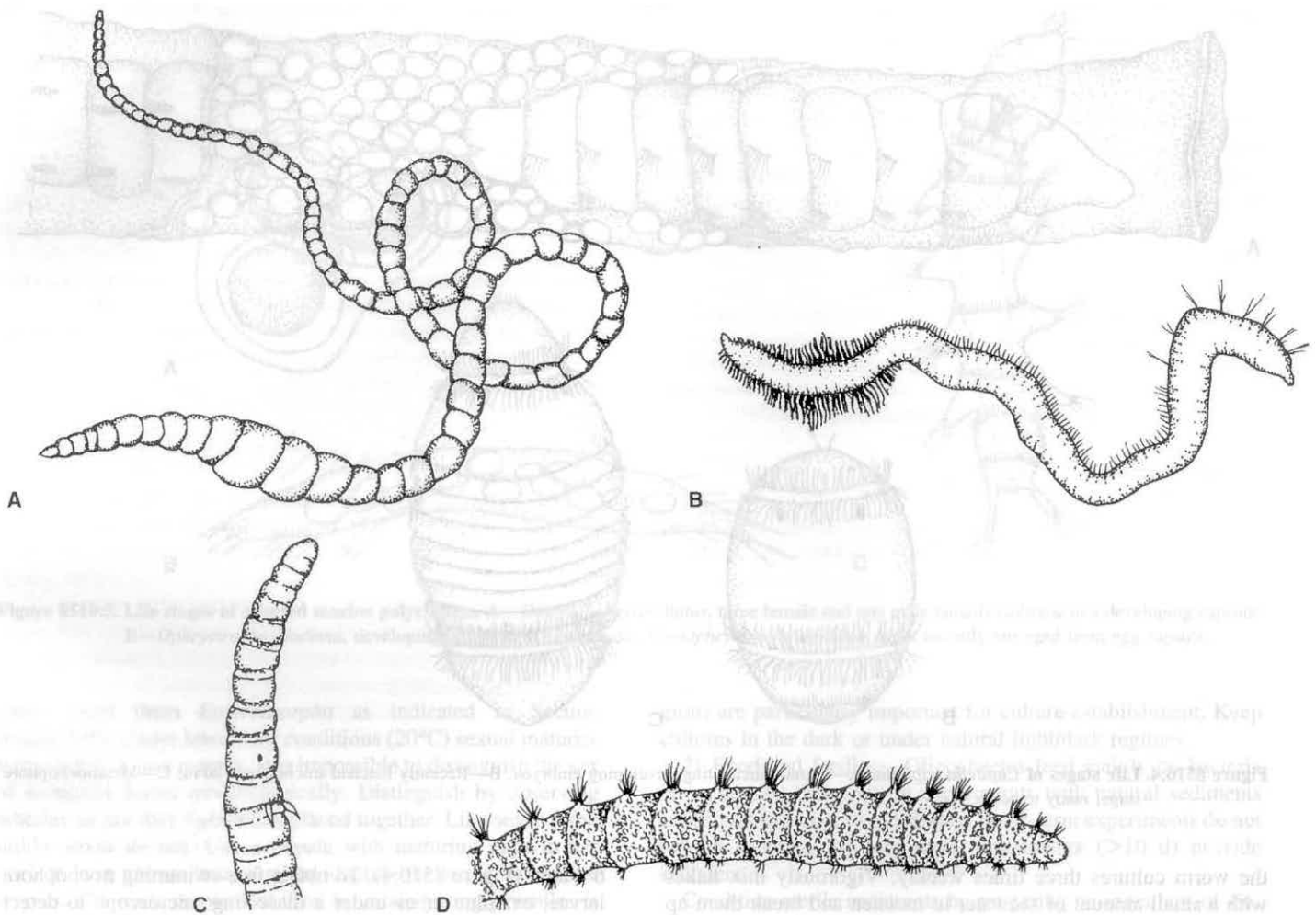


Figure 8510:3. Freshwater oligochaetes. A—*Tubifex tubifex*, adult; B—*Branchiura sowerbyi*, adult; C—*Stylodrilus heringianus*, adult; D—*Quistadrilus multisetosus*.

The marine species *Monopylephorus cuticulatus*, *Tubificoides fraseri*, and *Tectidrilus verrucosus*, are found in muddy sediments and are separable based on size (*M. cuticulatus* > *T. fraseri* > *T. verrucosus*), setae (*M. cuticulatus* has occasional irregular hair setae), and color (*T. verrucosus* has a papillate skin and greenish tinge, the others are dark red). Positive identification requires examination of preserved specimens.

To obtain worms, sieve the sediments through a 0.5-mm sieve and sort specimens under a dissecting microscope. Discard damaged worms. Transfer uninjured specimens to aerated aquariums or shallow trays for holding and feeding. Ensure that worms are collected from an uncontaminated area because rapid resistance to some toxicants can occur.¹

3. Culturing

a. Marine polychaetes:

1) Condition of animals—Discard animals injured during collection. Some species such as *Neanthes arenaceodentata* can regenerate a tail; thus it is not always necessary to discard worms missing tails when establishing a culture. Save worms with

gametes in the coelom for starting cultures, but normally do not use them for toxicity tests.

2) Food and feeding—Cultures of the polychaete species mentioned here can be maintained without sediment; therefore, the worms must be fed, as should worms in long-term experiments (see 8510C.4a). Cultures of the larger species (i.e., *N. arenaceodentata*) have better survival, growth, and tube production if fed a mixed diet that consists of a macroalga for tube construction and a commercially prepared invertebrate or fish diet for nutrition. The green alga, *Enteromorpha* sp., is convenient because it grows abundantly in most estuarine areas of North America. Collect in quantity, wash with seawater, dry, and store indefinitely. Before use, soak the alga in seawater and knead to separate individual filaments. Other macroalgae, for example, cultured brown *Ectocarpus siliculosus*, produce excellent results in polychaete cultures but are not as convenient to use. Place sufficient macroalga in culture containers to allow worms to construct tubes. Add commercially prepared diet* to

* Prawn Flakes, Plankton Flakes, TetraMarin, or equivalent.

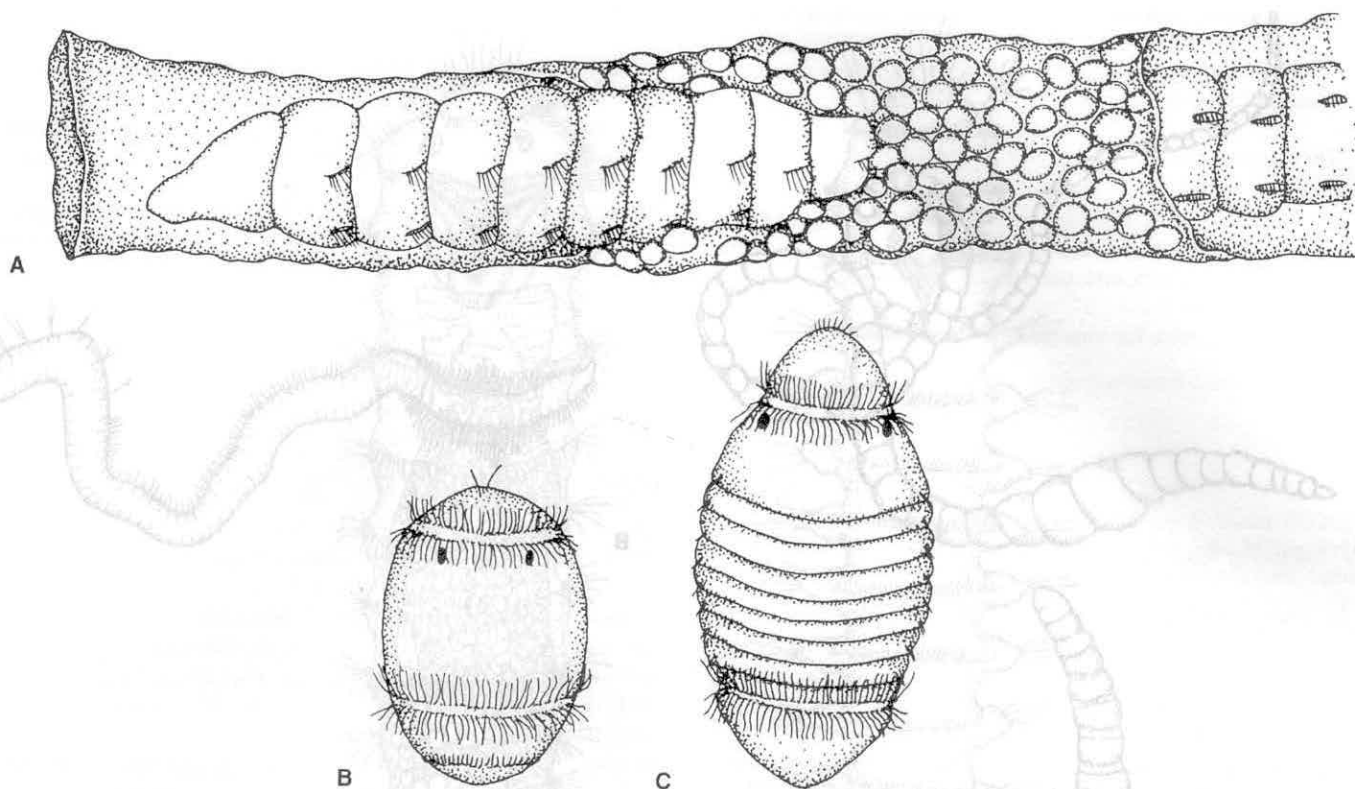


Figure 8510:4. Life stages of *Capitella capitata*. A—Female incubating developing embryos; B—Recently hatched trochophore larva; C—Metatrochophore stage, ready to settle.

the worm cultures three times weekly. Vigorously mix flakes with a small amount of seawater to moisten and break them up before adding them to the cultures. To minimize overfeeding, examine each culture container before adding the commercial diet. If most of the diet material is uneaten, do not add more and add less food at subsequent feedings.

A powdered diet is suitable for small species (*Ctenodrilus serratus*, *Capitella capitata*, *Ophryotrocha diadema*, and *Dinophilus gyrociliatus*) and the larvae of *N. arenaceodentata*. Prepare a fine powder from dried *Enteromorpha* sp. or one of the commercial diets by grinding the dry material in a blender and sieving it to smaller than 0.061 mm. *C. serratus* can be fed at a rate of 0.1 mL/worm/week of a mixture of 1.0 g powder/100 mL seawater.

Feed living *Dunaliella* sp. to larval *N. succinea* until the larvae settle. For culturing instructions see Section 8010E.4c1)b). Feed *Dunaliella* sp. at a minimum of 2 000 000 cells/L of worm culture or at a rate great enough to maintain a green color in the seawater. After the larvae settle, feed *Enteromorpha* sp. until the swimming reproductive epitoke stage is reached.

3) Producing test organisms

a) *Capitella capitata*—Laboratory-cultured specimens begin to mature in about 15 to 25 d after hatching. A mature female develops white masses of eggs in the coelom from about segment 10 posteriorly and a mature male develops specialized setae on the dorsal surface of segments 8 and 9. The female lays fertilized eggs along the inside lining of her tube where larval development continues until the trochophore larvae emerge 4 to

6 d later (Figure 8510:4). To obtain free-swimming trochophore larvae, examine tubes under a dissecting microscope to detect those containing eggs or larvae. Recently fertilized eggs appear white, but as they mature they become grey-green and can be seen moving about. Place tubes containing larvae in a petri dish. Under a dissecting microscope open the tubes to free the trochophores. One female provides 200 to 300 trochophores. Remove and discard the female and the tube containing any larvae that did not swim free. Use the free-swimming larvae in tests or let them develop for later use.

Sibling species of *Capitella capitata* have been described;² however, the taxonomic status of this species complex is still in question.

b) *Neanthes succinea*—Take nearly mature epitokes from the field or laboratory colony and hold until they complete sexual metamorphosis. Mature epitokes swim to the water surface and release gametes. If fertilization is successful, separate the zygotes into several 4-L jars containing aerated water and let them develop to the three-setiger stage (about 1 week). These larvae are ready for use in tests. One fertilization provides more than 2000 larvae.

c) *Neanthes arenaceodentata*—Before spawning, either the male or female enters the tube or burrow of another worm. If the worms are of different sex, they remain together and spawn within the tube. The female dies within 1 d after spawning and the male incubates the eggs for about 3 weeks, at which time they have 18 to 21 setigerous segments. At that time the young worms leave the tube, begin feeding, and construct their own

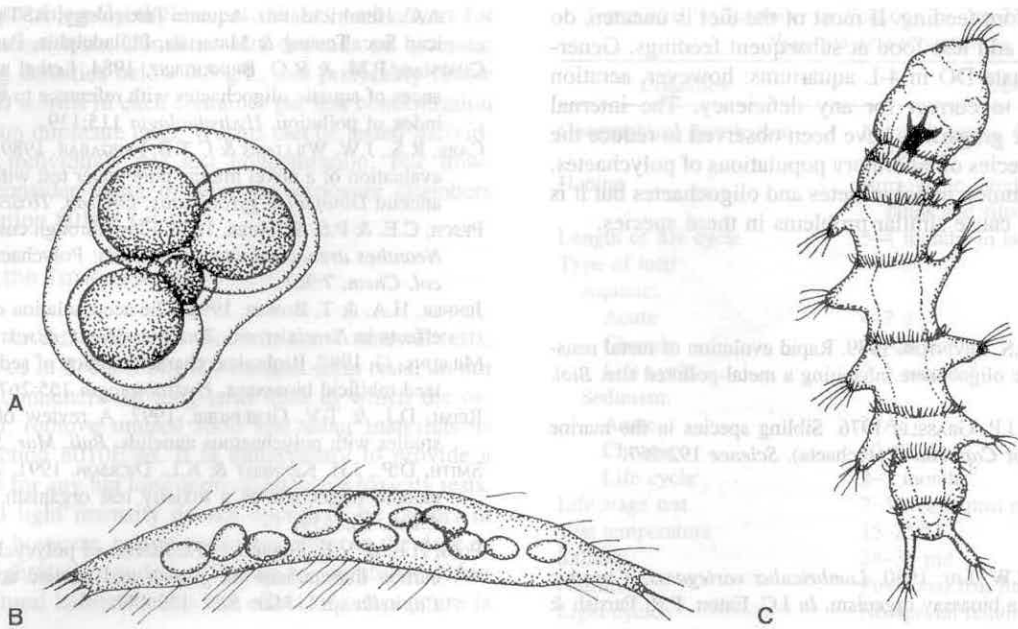


Figure 8510:5. Life stages of selected marine polychaetes. A—*Dinophilus gyrotilatus*, three female and one male (small) embryos in a developing capsule; B—*Ophryotrocha diadema*, developing embryos in egg capsule; C—*Ophryotrocha diadema*, larva recently emerged from egg capsule.

tubes. Feed them *Enteromorpha* as indicated in Section 8510B.3a2). Under laboratory conditions (20°C) sexual maturity is reached in 3 to 4 months. It is impossible to distinguish the sex of immature forms morphologically. Distinguish by observing whether or not they fight when placed together. Like sexes fight; unlike sexes do not. Use a female with maturing eggs in her coelom as a known individual to identify the sex of immature worms. The most convenient time to obtain young juveniles is shortly after they have left the parent's tube and have begun to feed.

d) *Ctenodrilus serratus*—This species reproduces asexually about every 14 d at 20°C by transverse division. Each individual produces about five to eight new specimens. Large colonies can be maintained with minimum care.

e) *Dinophilus gyrotilatus* completes its life cycle in 7 to 10 d at 20°C. The female lays two to five eggs in a capsule. The larger eggs develop into females and the smaller ones into males. The male mates with the female before hatching from the capsule (Figure 8510:5A), then dies. Large colonies can be maintained with a minimum of care.

f) *Ophryotrocha diadema* (Figure 8510:2B) completes its life cycle in 30 to 40 d at 20°C. This species is hermaphroditic with Segments 3 and 4 containing the male elements. The remaining segments posterior to Segment 4 are female. Eggs are laid in a loose jelly capsule (Figure 8510:5B) and number 10 to 14. They hatch from the capsule in 8 d as a four-segmented larva (Figure 8510:5C). They begin feeding the next day. Large colonies can be maintained with a minimum amount of care; however, subcultures should be established every 5 to 6 weeks in clean containers.

b. Freshwater and marine oligochaetes:

1) Condition of animals—Oligochaetes show great regenerative abilities; hence it is not always necessary to discard injured specimens. Mature individuals with well-developed clitellar re-

gions are particularly important for culture establishment. Keep cultures in the dark or under natural light/dark regimes.

2) Food and feeding—Oligochaetes feed mainly on bacteria in sediments; therefore, in experiments with natural sediments additional feeding is unnecessary. Short-term experiments do not require feeding; for long-term experiments (>10 d) provide sediment.

Condition sterile sediments by preparing an inoculum of *Enteromorpha* (for marine worms) or lettuce (for freshwater worms) consisting of the aqueous material remaining after decay of the plant fibers in diluent water. Add inoculum directly to culture containers in a volume not to exceed 10% of the total. Preferably use sediments of fine sand with some silt content, rather than more muddy sediments in which the worms are difficult to find. Check cultures periodically for spoilage; if this occurs, clean cultures and restart. Oligochaetes have no larval stage. No separate feeding regime is required for juveniles.

3) Producing test organisms—Gravid worms in culture lay eggs that hatch in 3 to 14 d, depending on species and temperature. Newly hatched worms lack the full complement of adult setae but rapidly develop these.

Freshwater species generally grow better in mixed culture. The following combinations are recommended: *L. hoffmeisteri* and *T. tubifex*, *L. hoffmeisteri* and *B. sowerbyi*, *T. tubifex* and *B. sowerbyi*, *S. heringianus* and *L. hoffmeisteri*. *T. fraseri* is a parthenogenic species and is particularly amenable to culturing; *T. verrucosus*, *L. variegatus*, and *M. cuticulatus* can be cultured as pure species.

4. Parasites and Diseases

Microbial growth can result from overfeeding, improper conditioning of food, or insufficient DO. Prevent fungal growths by providing sufficient aeration. To minimize overfeeding, examine

each aquarium before feeding. If most of the diet is uneaten, do not add more and add less food at subsequent feedings. Generally there is adequate DO in 4-L aquariums; however, aeration can be increased to correct for any deficiency. The internal protozoan parasitic gregarines have been observed to reduce the vitality of some species of laboratory populations of polychaetes. Gregarines are common in polychaetes and oligochaetes but it is not known if they cause similar problems in these species.

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8510 C. Toxicity Test Procedures

1. General Procedures

Use exploratory tests (see Section 8010D) to determine toxicant concentrations for short-term tests. Prepare dilution water and toxicant solutions and introduce them into test containers as described in Section 8010F.

2. Water Supply

a. *Artificial seawater*: See Section 8010E.4b2). Use a salinity of approximately 35.5 g/kg and a pH of about 7.8 for marine populations; use lower salinity for estuarine worms.

b. *Natural seawater*: Determine and report quality routinely. Maintain dilution water salinity at or near selected or normal concentration. During a test, do not allow salinity to vary by more than ± 3 g/kg. Filter seawater through a 0.45- μ m membrane filter.

c. *Distilled or tap water*: Determine quality (hardness, alkalinity, chemical constituents) and report routinely. Use a near-neutral (pH 7.0) water.

3. Exposure Chambers

a. *Marine polychaetes*: Use 4-L aquariums or glass jars for short-term and intermediate static and renewal tests and for long-term tests where flow-through facilities are not appropriate. Cover aquariums to prevent entrance of foreign materials. Do not add more than 2.5 L test solution to each 4-L aquarium. Use 500-mL erlenmeyer flasks, containing 100 mL seawater, for either short-term or long-term experiments when only one organism is placed in each flask. Close the flask with a No. 7 TFE stopper fitted with a glass tube for aeration. Use small stender dishes (30

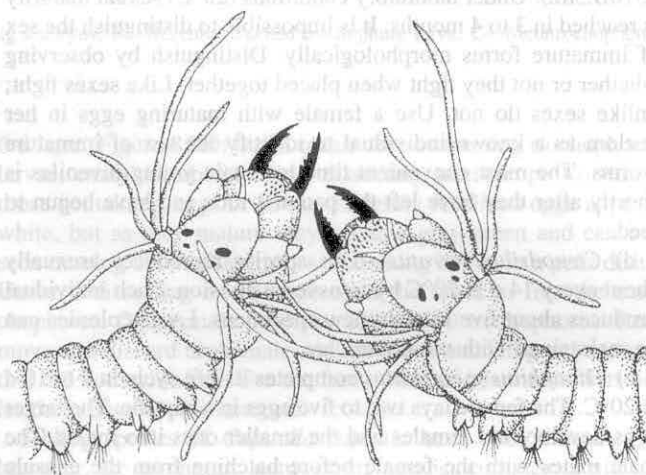


Figure 8510:6. *Neanthes arenaceodentata*. Adults of same sex in a fighting position.

mL) for larval tests. For flow-through tests, use exposure chambers described in Section 8010F.1c). In the case of cannibalistic species such as *Neanthes arenaceodentata* (Figure 8510:6), isolate individuals during testing. Container size depends on biomass; maintain loading densities below 0.5 g/L for static conditions and below 0.5 g/L/d for flow-through tests at 20°C. For tests with sediment, use glass crystallizing dishes of the appropriate size for species tested and number of individuals per dish. Fill dishes with sediment 1 to 4 cm deep. Let clean seawater flow over top of sediment.

b. *Freshwater and marine oligochaetes*: Conduct test in a manner similar to that described for the polychaete larval tests.

Use shallow disposable polyethylene petri dishes with covers for static or replacement tests. Container size depends on biomass; maintain loading densities below 0.5 g/L, and preferably below 0.2 g/L. Place 10 worms in each container per test concentration plus controls. Run duplicate tests. Worms can be tested individually, with 20 individuals per test concentration. For flow-through tests, consider using or adapting exposure chambers described in Section 8010F.1c).

4. Conducting the Toxicity Tests

a. Setting up the test chambers: For static and renewal tests, set up as described in Section 8010D. In short-term tests, do not clean exposure containers. In long-term tests in which the organisms are fed, remove unused food and other materials as described in Section 8010E.4d. It is unnecessary to provide a bottom substrate for any but long-term oligochaete toxicity tests. Photoperiod and light intensity do not appear to be factors in polychaete tests; however, test oligochaetes either in the dark or with a natural light/dark simulation.¹ Keep temperatures within $\pm 2^\circ\text{C}$ of the natural habitat unless the effect of temperature is being tested.

1) Marine polychaetes—Use a minimum of 20 worms for each test concentration. For cannibalistic species, such as *N. arenaceodentata*, use one worm per container. For other species, place 2, 5, or 10 worms in each container (depending on biomass and container size, see Section 8010F.3c). For tests with sediments, use a minimum of three replicate dishes per sediment concentration and a minimum of five worms per dish. Individuals of cannibalistic species do not have to be separated with sediment in the dishes. Table 8510:I summarizes the ecological and test conditions for *Neanthes arenaceodentata*.

2) Freshwater and marine oligochaetes—Use a minimum of 20 worms for each test concentration, preferably in two replicates of 10 worms each. Although the worms will intertwine when healthy, toxified individuals remain separate and toxic effects (e.g., the progressive disintegration of posterior segments) will be manifest.

b. Duration and type of test:

1) Short-term tests—The length of short-term or acute tests depends on the length of the organisms's life cycle (see Section 8010F.3a). Short-term tests may be conducted under static conditions (recommended for tests of shorter duration, i.e., <4 d) or with periodic renewals for tests of longer duration (i.e., >4 d).

2) Intermediate-length tests—Use tests of intermediate length for determining adult polychaete survival. For most species conduct these renewal or flow-through tests for 20 to 28 d.

3) Long-term tests—Long-term tests are either partial- or full-life-cycle tests. Partial-life-cycle tests begin with the polychaete trochophore larval or oligochaete egg-case stage and end with sexual maturity. Full-life-cycle tests also begin with larvae or egg-case stage but continue through reproduction and subsequent egg production or larval settlement of the offspring. Because of the long duration, conduct these tests using either periodic renewal or flow-through conditions.

Select and prepare test concentrations as described in Section 8010F.2b. Measure and mix dilution water and stock toxicant solutions by proportional diluters and deliver to exposure chambers as described in Section 8010F.1. Make tests in flow-through exposure chambers similar to those described in Sections

TABLE 8510:I. SUMMARY OF ECOLOGICAL AND TEST CONDITIONS FOR *NEANTHES ARENACEODENTATA*

Condition	Description
Geographical distribution	North America, Europe, South Pacific Ocean ³
Habitat	Burrows in intertidal and subtidal sands and silts ³
Length of life cycle	3–4 months in laboratory ³
Type of test:	
Aquatic:	
Acute	4–7 d ³
Chronic	10–28 d
Life cycle	2–3 months ³
Sediment:	
Acute	N/A
Chronic	10–28 d ⁵
Life cycle	2–3 months ⁴
Life stage test	2–3 weeks post emergence ⁴
Test temperature	15–25°C
Salinity	28–36 ppt
Nutrition	Powdered fish flakes ⁵
Light cycle	No special requirement
Control mortality	Not to exceed 10%
End points:	
Acute	Death
Chronic:	
Aquatic	Death
Sediment	Growth as determined by dry weight
Life cycle	Number of offspring
Reference toxicant (CdCl ₂)	14.1 $\pm$ 1.2 mg/L 96-h LC50

8740C.3 and 8010F.1). Renewal tests using up to 4-L exposure chambers may be necessary if flowing water is unavailable.

The duration of long-term tests depends on length of life-cycle of the organism. For example, for polychaetes it varies from about 1 month with *C. capitata* to 3 or more months with *N. succinea*, *N. virens*, and *N. arenaceodentata*.

c. Test organisms: See Section 8510B.

d. Performing tests:

1) Short-term tests—Set up and conduct renewal tests as described in Section 8010D.2. Determine survival of adults by checking exposure chambers at 1, 2, 4, 8, 18, and 24 h, then once or twice daily thereafter. Dead specimens generally are pale and swollen and lie on the bottom; live specimens usually respond to physical stimulation. If the tests are more than 4 d long, renew solutions, preferably daily but at least every fourth day. In short-term tests with polychaete larvae, determine survival after 96 h by microscopic examination. The absence of larvae generally indicates death because decomposition of small larvae is rapid.

2) Intermediate-length tests—Set up test chambers described in ¶ 4a above to determine adult lethality (LC50 or incipient LC50). Examine test containers daily to determine survival. If no organisms are killed after a certain length of exposure, report the period beyond which there is no further kill and the percentage killed in each test concentration. For contaminated sediment tests, sieve contents of each replicate dish and count number of survivors. If a graded series of mixed sediments has been used, calculate LC50 based on percentage of contaminated sediment.

3) Polychaete life-cycle tests beginning with the trochophore larval stages—Set up as described previously in this section. Conduct test through sexual maturity with test periods varying

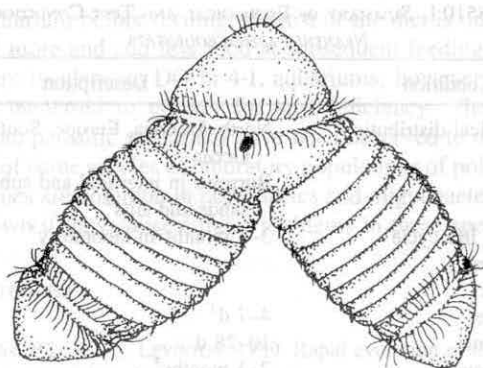


Figure 8510:7. *Capitella capitata*. Abnormal larva.

from 3 to 4 weeks with *C. capitata*, and from 2 to 3 months or longer for *N. arenaceodentata*, *N. succinea*, and *N. virens*. Feed larvae as described in Section 8510B.3a2). Determine survival at least twice weekly for *C. capitata* and once a week for *Neanthes*. During the early part of the study, count organisms on bottom of exposure chambers. If a renewal test is being conducted, decant supernatant fluid, examine under a dissecting microscope, and replace fluid with fresh test solution. For flow-through tests, remove chambers from exposure box, count organisms, and replace chamber. If no organisms are observed by the third examination, terminate that test chamber.

When *C. capitata* is the test organism, remove test chambers after about 15 to 16 d and every 2 d thereafter to check with a dissecting microscope for presence of eggs in the coelom and later for the presence of zygotes along the sides of the tube. Remove females when developing eggs are in the trochophore stage and count the larvae. Discard females and larvae after counting larvae and record the number of dead and deformed larvae. Continue to examine each exposure chamber every 2 d for detection of females incubating larvae until all females have been removed and the total number of larvae recorded.

Abnormal larvae of *C. capitata* (Figure 8510:7) have been observed during life-cycle tests when exposed to sublethal concentrations of chromium, zinc, or detergents.²

For *N. succinea*, set up exposure chambers as described in ¶ 4a above with 25 larvae in each 1-L exposure chamber or 10 larvae in each flow-through exposure chamber. Use 10 chambers per concentration tested. Because these worms fight and are cannibalistic when crowded, prepare additional exposure chambers or reduce numbers in each test chamber to five individuals after the first month. Continue tests until animals reach the epitoke stage; then determine individual lengths and total weights and compare with those in the control.

4) Polychaete life-cycle tests beginning with the newly settled larval stage—These tests will vary in duration from about 1 month for *C. capitata* to 3 months or more for *N. arenaceodentata*. Set up tests as described previously with newly settled larvae. Use a minimum of two specimens per flask and 10 flasks per concentration. As tests progress, count organisms as above. Examine for survival once or twice per week as in ¶ d3).

For *N. arenaceodentata*, use recently emerged juveniles having approximately 18 to 21 setigerous segments. If a renewal test is conducted, place four worms in each of five 4-L exposure chambers with 2.5 L test solution. Set up five containers for each

test concentration and control. For flow-through tests, place two larvae in each of 10 exposure chambers for each test concentration and the controls. At 25 d, examine worms by viewing from outside the container for the presence of eggs in the coelom. If necessary, move mature worms among the replicates of a given treatment to pair males with females. Mature eggs reach 450 µm diam and are yellowish-orange. Examine at 5-d intervals until eggs are noted and then at 2- to 3-d intervals to determine whether eggs are being laid. The females die within 1 d after laying eggs and the males incubate them for about 3 weeks. The life cycle is complete when the juvenile worms emerge from the parental tube. Remove males and count larvae.

5) Oligochaete life-cycle tests—Set up as described above. Test duration depends on test conditions and end points chosen. Use procedures similar to those for polychaetes.

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8510 D. Sediment Test Procedures Using the Marine Polychaete *Neanthes arenaceodentata*

1. General Procedures

Before conducting a definitive sediment test with the polychaete *Neanthes arenaceodentata*, conduct preliminary tests to become familiar with the test procedures given below. See Table 8510:I for a summary of ecological and test conditions for this species.

2. Water Supply

a. *Artificial seawater*: See Section 8010E.4b2). Use a salinity of approximately 28 to 35 g/kg and a pH of approximately 7.8.

b. *Natural seawater*: Determine and report salinity routinely. During the test, do not let the salinity vary more than ± 3 g/kg.

3. Sediment

a. *Collection*: Collect sediment with a benthic grab such as a van Veen bottom sampler [see Section 10500B.3a3)]. Collect multiple samples at a site to obtain the required 25 L of sediment. Press sediments through a 1.0-mm sieve, using a minimum amount of water, to remove macroscopic invertebrates. Place all sieved sediments in a plastic bucket and mix later in the laboratory. Hold sediments at 4°C until start of test; use within 2 weeks of collection, if possible. Collect uncontaminated or reference sediments from an area with similar-sized particles free from contaminants.

b. *Sediment chemistry*: Analyze sediments for grain size, total organic carbon, metals, and organic compounds.

4. Exposure Chambers

Use as a test chamber a 1-L glass beaker with an internal diameter of 10 cm. Cover beakers with a watch glass to minimize evaporation and to reduce contamination. Aerate each chamber through a 1-mm-opening glass pipet that extends between the beaker spout and the watch glass cover to a depth of not closer than 2 cm from the sediment surface. See Figure 8510:8.

5. Conducting the Sediment Toxicity Test

a. *Setting up the test chambers*: Use five replicates with each sediment sample. Add sufficient sediment to each beaker to make a 2.0-cm layer. Carefully add approximately 750 mL sea water to each beaker with minimal physical disturbance of the sediment. Aerate at the rate of about 1 to 2 bubbles/s. Prepare sediments and overlying water the day before starting the test to provide time for sediment and seawater to adjust to test temperature.

b. *Environmental conditions*: Although photoperiod is not critical, usually make sediment tests with *Neanthes arenaceodentata* at either 16 h light/8 h dark or 12 h light and dark. Make tests at $20 \pm 2^\circ\text{C}$.

c. *Test animals*: The male *N. arenaceodentata* incubates the developing embryos in his tube for approximately 3 weeks after fertilization. Embryos do not feed during this time but subsist on

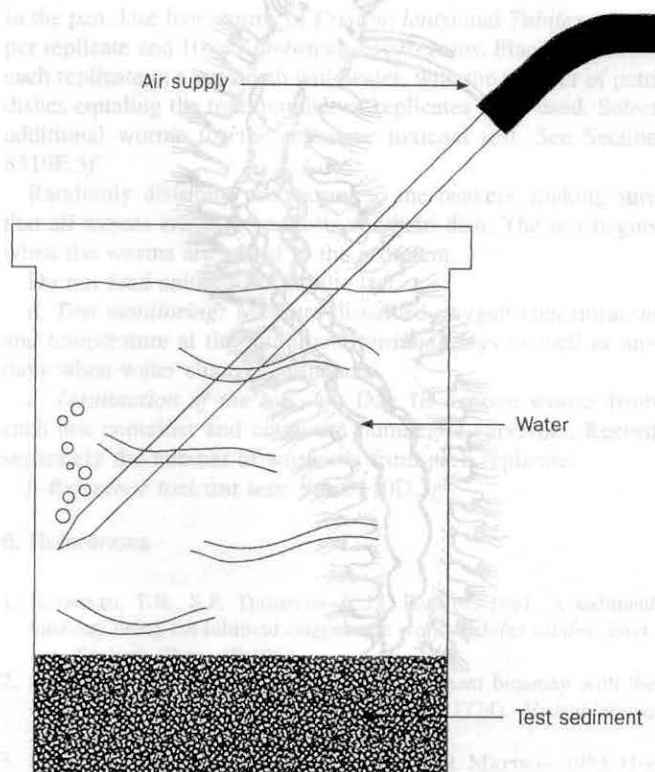


Figure 8510:8. Experimental setup for sediment testing.

the yolk within the embryo. At 21 to 24 d the juvenile worms leave the tube of the male parent and begin to feed. In the sediment test, use juvenile worms (Figure 8510:9) that are 2 to 3 weeks post-emergent or are approximately 5 to 6 weeks of age from the time of fertilization. See Section 8510B.3a3)c). Place all juvenile worms to be used in a test together in a white, enamel pan and select uniform-sized worms. Generally place 20 to 30% more worms than needed in the pan and discard the larger and smaller ones. Place five worms in a petri dish with seawater with the number of petri dishes equaling the total number of replicates to be used in the test plus five additional dishes each with five worms. Choose five dishes at random and weigh the five worms together to obtain initial dry weight. See Section 8510D.5e. Select additional worms for the reference toxicant test. See Section 8510D.5f.

Randomly distribute the worms to the beakers, making sure that all five worms are removed from the petri dish. The test begins when the worms are added to the sediment.

Add 5 mL food slurry solution to each test container every other day. To make this solution, grind food* into a fine powder and mix with seawater at a ratio of 1.0 g food to 25 mL seawater. On days when feeding and water changes occur on the same day, add food after the water change.

* TetraMarin® or equivalent, widely available in aquatic pet supply stores.

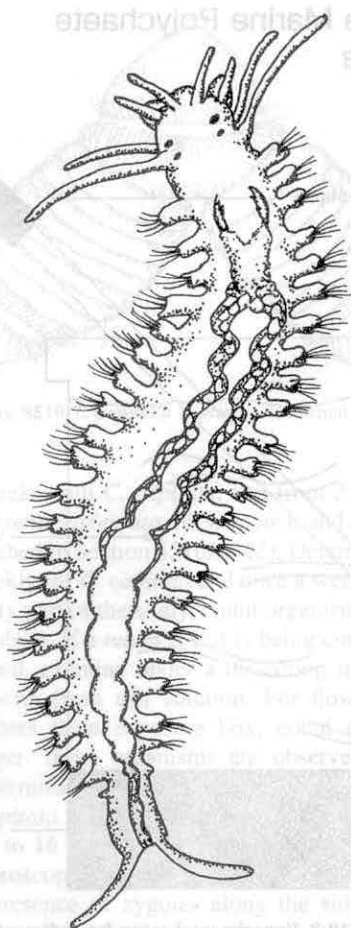


Figure 8510:9. *Neanthes arenaceodentata*. Larva recently emerged from male parent's tube.

d. Test monitoring: During the test, examine beakers daily to ensure that aeration is adequate. On Days 3, 6, 9, 12, 15, and 18 for the 20-d test, replace one half of the seawater within each beaker with clean seawater. Measure dissolved oxygen, temperature, and salinity on the initial and terminal days and the days specified above before the water change.

e. Termination of the test: On Day 20 remove worms from each test container, count, place in a clean small petri dish, and wash in distilled water. Place worms from each replicate in a pre-weighed aluminum pan and dry at 50°C until constant weight (overnight); record dry weight of worms in each pan. Record as the dry weight per worm per day as calculated using the formula:

$$G = (W_t - W_i) / T$$

where:

G = estimated individual growth rate, mg dry weight/d,

W_t = mean estimated individual dry weight, mg, at termination of test,

W_i = mean estimated individual dry weight, mg, at start of test, and

T = exposure time, d.

The use of the mean individual growth rate per worm per day facilitates comparison of results between different sediments tested and with other experiments. Because each test is not started with exactly the same size worm (weight), expressing growth as a rate function rather than absolute mass per worm normalizes results.

f. Reference toxicant test: Make a reference toxicant test, or positive control, concurrently with the sediment test and use it to check the health of the test animals. Use reference test animals from the same batch as those used in the sediment test. Cadmium chloride, expressed as cadmium, is commonly used as the reference toxicant; however, other chlorides of metals or organic compounds may be used. The reference toxicant test is a standard 96-h test without sediment.

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8510 E. Sediment Test Procedures Using the Freshwater and Marine Oligochaetes *Pristina leidyi*, *Tubifex tubifex*, and *Lumbriculus variegatus*

1. General Procedure

The following procedures comprise short-term acute tests. Oligochaetes also can be used to measure reproductive effects^{1,2} and in combined toxicity and bioaccumulation tests.³

Before conducting a definitive sediment test with the oligochaetes, *Pristina leidyi*, *Tubifex tubifex*, or *Lumbriculus variegatus*, conduct preliminary tests to become familiar with the test procedures given below.

2. Water Supply

Use fresh water for all tests. It may be moderately hard synthetic water prepared with a commercially available system,* deionized water and reagent-grade chemicals, receiving water, or synthetic water modified to reflect receiving water hardness.

3. Sediment

Collect sediment with a benthic grab such as an Ekman or van Veen sampler. See Sections 10500B.3a3) and 6) and 8510D.3a and b.

4. Exposure Chambers

Use as a test chamber a 250-mL beaker with an internal diameter of 6 cm. Cover beakers with a watch glass to minimize evaporation and to reduce contamination. Aeration is unnecessary.

5. Conducting the Sediment Toxicity Test

a. *Setting up the test chamber:* See 8510D.5, but add only about 100 mL water to each beaker.

b. *Environmental conditions:* Set lighting for a 16 h light/8 h dark photoperiod at an intensity of 550 to 1050 lux (50 to 100 ft-c). Make tests with *Pristina leidyi* at $24 \pm 1^\circ\text{C}$ and at 20 to 25°C with *Tubifex tubifex* and *Lumbriculus variegatus*.

c. *Test animals:* Use specimens of mixed age in these tests. Place worms in a white, enamel pan and select healthy-appearing specimens. Generally place 20 to 30% more worms than needed

in the pan. Use five worms of *Pristina leidyi* and *Tubifex tubifex* per replicate and 10 of *Lumbriculus variegatus*. Place worms for each replicate in a petri dish with water, with the number of petri dishes equaling the total number of replicates to be used. Select additional worms for the reference toxicant test. See Section 8510E.5f.

Randomly distribute the worms to the beakers, making sure that all worms are removed from the petri dish. The test begins when the worms are added to the sediment.

Do not feed animals during the test.

d. *Test monitoring:* Measure dissolved oxygen concentration and temperature at the initial and terminal days as well as any days when water changes are made.

e. *Termination of the test:* On Day 10 remove worms from each test container and count the number of survivors. Record separately the number of survivors from each replicate.

f. *Reference toxicant test:* See 8510D.5f.

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* Millipore Milli or equivalent.

8510 F. Data Evaluation

1. Short-Term and Intermediate Adult Survival Studies

Determine the LC50 values for each exposure period as described in Section 8010G. A useful supplementary measure is to

determine LT50 (time to 50% mortality) in comparative exposures to single toxicant, effluent, water, or sediment concentrations. LT50s also provide useful ancillary information for LC50 studies.

2. Polychaete Life-Cycle Studies Beginning with the Trochophore and Settled Larval Stages

The number of females forming and laying eggs and the number of offspring produced are inversely related to sublethal toxicant concentrations at levels below the LC50. They provide a more subtle measure of effects than the LC50.

Record life-cycle data for each concentration of toxicant as follows: number of females forming eggs, number of females laying eggs, and number of eggs and live offspring produced. Compare these data, expressed on a percentage basis, for all test concentrations with those obtained from the controls. Use statistical and reporting techniques described in Sections 8010G and H.

8610 MOLLUSKS*

8610 A. Introduction

1. Characteristics and Ecology

The Phylum Mollusca, the second largest phylum of the animal kingdom, is made up of such forms as clams, mussels, oysters, snails, slugs, octopuses, squids, as well as others. The life cycle of mollusks varies from about 1 to about 10 years. In numbers of species (about 100 000 living and 35 000 fossil), mollusks are second only to the arthropods. Their ecology also is very diversified: they have been able to live successfully in nearly all terrestrial, freshwater, and marine habitats, from the greatest depths of the ocean to the highest altitudes recorded for animal life.^{1,2} Mollusks are important ecologically and are a source of food for human beings around the world.

termination, tissues can be excised and analyzed chemically for pollutants, such as pesticides and heavy metals, to determine whether animals have accumulated toxic substances above background levels.

A field test consists of placing freshwater or marine species of bivalves in cages and suspending them in the water column for 30 d or longer. End points may be survival, growth, or accumulation of toxic substances above background levels in tissues excised for chemical analysis.

Other procedures for toxicity tests using mollusks have been published.³⁻⁵

2. Types of Tests

Mollusks, particularly marine gastropods (snails and slugs) and bivalves (clams, mussels, oysters, etc.), have been used extensively as bioassay test species throughout the United States. Two types of larval development toxicity tests are described in this section. The marine bivalve larval development test is suitable for water toxicity testing for all coasts and uses oysters (*Crassostrea* spp.), mussels (*Mytilus* spp.), or quahog clams (*Mercenaria mercenaria*). Larvae of the red abalone, an edible marine gastropod (*Haliotis rufescens*), are used in bioassays on the Pacific Coast.

A second type of test exposes adult bivalves, such as bent-nose clams (*Macoma nasuta*), to sediments in the laboratory. At test

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* Approved by Standard Methods Committee, 2001.

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8610 B. Selecting and Preparing Test Organisms

1. Selecting Test Organisms

In accordance with the criteria listed in Section 8010E.1, the recommended test species include (but are not restricted to) the following:

a. Marine gastropods:

Family Haliotidae:

Haliotis rufescens (west coast of U.S.)

b. Marine bivalves:

Family Ostreidae (Section 10900, Plate 21:N):

Crassostrea gigas (west coast of U.S.)

Crassostrea virginica (east coast of U.S.)

Family Veneridae:

Mercenaria mercenaria (all of U.S. coasts) (Section 10900, Plate 21:J)

Family Tellinidae:

Macoma balthica (Atlantic Ocean)

Macoma nasuta (west coast of U.S.)

Family Mytilidae:

Mytilus spp. (cosmopolitan) (Section 10900, Plate 21:L)

c. Freshwater bivalves:

Family Unionidae

Elliptio complanata

Pygodon grandis

Family Corbiculidae

Corbicula fluminea

Family Sphaeriidae

Sphaerium simile (Section 10900, Plate 21:H)

Family Dreissinidae

Dreissena polymorpha (Section 10900, Plate 21:F)

d. Other freshwater and marine mollusks: Also used in toxicity tests, but not discussed, are the freshwater *Anodonta imbecillis* (Section 10900, Plate 21:E), and the marine species *Ostrea lurida*, *Ostrea edulis*, *Argopecten irradians irradians*, *Spisula solidissima*, *Mulinia lateralis*, *Perna* spp., and *Rangia cuneata* (Section 10900, Plate 21:M).

2. Collecting and Conditioning Test Organisms

a. *Marine gastropods*: Collect *Haliotis rufescens* (red abalone) adults from the field or purchase from commercial dealers. Mature red abalone can be collected on rocky substrates from the intertidal zone to depths exceeding 30 m. They are most commonly found in crevices where there is an abundance of macroalgae. State collection permits are always required for collecting abalone. While abalone captured in the wild can be induced to spawn, those grown or conditioned in culturing facilities have been more dependable. Commercial mariculture facilities can supply ripe organisms (sexually mature abalones are usually about 70 mm or more in shell length). In any case, obtain brood stock from sources free of contamination by toxic substances to avoid genetic or physiological preadaptation to pollutants. In the laboratory, identify the sex of the organism by inspecting the gonads, located under the right posterior edge of the shell. An abalone placed upside down on a flat surface will soon relax and begin moving the foot trying to right itself. During this movement, bend the foot away from the gonad area and determine the

sex. The ovary is jade green, the testes are cream-colored. When the gonad fully envelopes the dark blue-gray conical digestive gland and is bulky along its entire length, the abalone is ready for spawning.¹ Ripe spawners have a distinct color difference between the gray digestive gland and the green or cream-colored gonad. Less developed gonads appear gray (in females) or brown (in males).

b. Marine bivalves:

1) *Crassostrea gigas* (Pacific oysters), *Crassostrea virginica* (Eastern oysters), *Mercenaria mercenaria* (quahog clams), and *Mytilus* spp. (mussels) may be collected in the field, but those purchased from commercial suppliers who deal in bioassay organisms usually will spawn more consistently. Identify all field-caught bivalves to species.²

2) *Macoma nasuta* (bent-nose clams) commonly inhabit intertidal areas such as bays and harbors. They reside in the upper few millimeters of fine sediment, which may be collected by hand and sieved to acquire specimens. Identify field-caught organisms to species before their use in testing procedures. Animals also are commercially available. Hold clams as briefly as possible and introduce into test sediments soon after collection.

c. Freshwater bivalves:

1) Zebra mussels (*Dreissena polymorpha*) are the only freshwater bivalves that attach permanently to substrates with byssal threads. They were introduced into the Great Lakes and have spread to other freshwater localities. Because they attach themselves to substrate, they can be collected in large numbers.

2) Asian clams (*Corbicula fluminea*), also introduced into North American waters, occur in large numbers and are easily collected.

3) Fingernail clams (*Musculium transversum*) are small and are convenient test animals because of their size.

3. Culturing

a. *Marine gastropods*: Keep abalone separated in aerated tanks with flowing seawater. Ideal maintenance temperature is $15 \pm 1^\circ\text{C}$, the toxicity test temperature. If brood stock are to be held for longer than 5 d at the testing facility, feed brood stock with blades of the giant kelp, *Macrocystis* (Section 10900, Plate 21A,E). Feed to slight excess; large amounts of uneaten algae will foul culture water. If *Macrocystis* is unavailable, substitute other brown algae [*Nereocystis* (Section 10900, Plate 2A:G), *Egria*, *Eisenia*] or any fleshy red alga.¹ For brood stock, preferably use abalone 7 to 10 cm in shell length. They are easier to handle than larger ones, and can be spawned more often (about every 4 months under suitable culture conditions).³

b. *Marine bivalves*: Maintain adult bivalves in glass aquaria or fiberglass tanks and continuously supply with high-quality, fresh seawater (salinity 18 to 34 g/kg, depending on location and species). If a continuous flow-through system is not available, use natural seawater or commercially available sea-salt preparations made with deionized water in a recirculating system. Water may require efficient activated charcoal or other type of filtration to maintain high water quality. Observe animals daily, and discard any obviously unhealthy animals. Before spawning or

direct use in a toxicity test, brush or gently scrape animals to remove barnacles or other encrusting organisms. If conditioning bivalves to spawn, hold for 1 to 8 weeks at 20°C for oysters and quahogs and 15 to 18°C for mussels. During extended holding and conditioning, provide animals with an adequate supply of natural or cultivated phytoplankton to ensure adequate nutrition. Alternatively, some suppliers will precondition animals before shipping. Animals then may be received on the same day as the test, and the maintenance of an elaborate seawater holding system can be avoided.

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8610 C. Short-Term Test Procedures Using Marine Mollusk Larvae

1. General Procedures

Use exploratory tests (see Section 8010D) to determine toxicant concentrations for short-term tests. Prepare dilution water and toxicant solutions and introduce them into test containers as described in Section 8010F.

2. Water Supply

a. Marine gastropod larvae: Tests require a marine laboratory with a supply of clean, 20- μ m-filtered seawater, with salinity 34 $\pm$ 2 g/kg.

b. Marine bivalve larvae: Use high-quality natural seawater, preferably filtered to 20 μ m. Test salinity (18 to 34 g/kg) is dependent on the location and species selected for the test, but regardless of selected salinity, do not vary the salinity by more than $\pm$ 1 among treatments.

3. Exposure Chambers

Use 30- to 600-mL borosilicate glass beakers or nontoxic disposable containers, five chambers per concentration. Cover the chambers during the test with glass plates or individual caps to avoid contamination from air and excessive evaporation of test solutions. Soak new test chambers in dilution water overnight.

4. Conducting the Toxicity Tests

a. Spawning and fertilization: If mature brood stock are shipped by a supplier, allow 2 d or more for laboratory accli-

mation before spawning induction; this should increase the probability of achieving a successful spawn of viable gametes. Always bring brood stock up to acclimation temperature slowly to avoid premature spawning. Before beginning the spawning induction process, be sure that test solutions will be mixed, sampled, and temperature-equilibrated in time to receive the newly fertilized eggs. Spawning induction generally takes about 3 h, but if embryos are ready before the test solutions are at the proper temperature, the delay may allow embryos to develop past the one-cell stage before transfer to the toxicant. Transfer can then damage the embryos, leading to unacceptable tests results.

1) Marine gastropod larvae—Ripe abalone can be induced to spawn by stimulating the synthesis of prostaglandin-endoperoxide in the reproductive tissues.¹ This can be done by addition of hydrogen peroxide to seawater buffered with tris(hydroxymethyl)aminomethane (Tris)¹ or by irradiation of seawater with ultraviolet light.² Select four ripe male and four ripe female abalone. Place organisms of each sex in separate, clean polyethylene buckets filled with 6 L of 0.22- μ m-filtered seawater. Aerate the water and keep at 15°C. Prepare buffer by placing 12.1 g Tris in a 125-mL erlenmeyer glass flask and adding 50 mL deionized water. Mix solution thoroughly until the buffer reagent is completely dissolved. Then, into a flask, mix 12 mL of 30% H₂O₂ with 38 mL deionized water. Use hydrogen peroxide less than 1 year old from purchase date, refrigerated, and opened for no more than 30 d.

Add 25 mL buffer solution to each bucket followed by 25 mL H₂O₂ solution; mix thoroughly. Keep buckets covered and un-

disturbed for next 2.5 h of exposure. After exposure, empty, rinse, and refill buckets with 6 L 0.22- μ m-filtered seawater. Continue moderately heavy aeration until spawning begins. Siphon eggs into 1-L glass beaker fertilization container. Fertilize eggs within 1 h of release by adding 100 to 200 mL sperm-laden water. Use a gentle flow of filtered seawater poured along the edge of the fertilization container to roil the eggs, mixing them with sperm. Let fertilized eggs settle for 15 min before siphoning off the sperm-laden water. Refill container with filtered seawater and settle eggs again. After 15 min, siphon fertilized eggs into a 1-L beaker for counting. Evenly mix the embryos in the 1-L beaker by gentle vertical stirring with a clean perforated plunger.³ Never let embryos settle densely in the bottom of the beaker, and take care not to crush embryos while stirring. Using a 1-mL wide-bore graduated pipet, take five samples of evenly suspended embryos. Empty contents of pipet onto a Sedgwick-Rafter slide and count embryos. Take the mean of five samples to estimate number of embryos. Number of embryos in the beaker should be between 200 and 300 embryos/mL. Dilute if concentration is too high, let embryos settle and pour off excess water if concentration is too low.

2) Marine bivalve larvae—Remove at least a dozen bivalves from conditioning chamber and place them in a container filled with seawater at about 20°C for oysters and quahogs and 15°C for mussels. They should resume pumping within about 30 min. Over a 15- to 20-min period, slowly increase the temperature; do not exceed 32°C for oysters and quahogs and 20°C for mussels. If animals do not spawn within 30 min, return them to water at the original temperature, and, after about 15 min, raise temperature again. Other methods have been used successfully to induce bivalves to spawn. The addition of algae into the water may work for all bivalves. The injection of the posterior adductor muscle with 0.5M potassium chloride has been used successfully for mussels, and the addition of heat-killed sperm has worked for oysters.

1) When individuals are observed to be shedding gametes, place each spawning bivalve in an individual chamber containing seawater at 20°C for oysters and clams and 15°C for mussels. Examine a small sample of gametes from each spawner. Although it is highly desirable to pool populations of sperm and eggs, it is more important that they are of high quality. Use only sperm that are highly motile and eggs that are not vacuolated, small, or abnormally shaped. Combine the best sperm and filter through a 36- μ m screen* to remove extraneous material or clumped gametes. Filter the eggs through a 75- μ m nylon screen into a 1-L beaker and dilute to a density of 5000 to 8000 eggs/mL. Use a Sedgwick-Rafter slide to verify density. While using a perforated plunger to continually suspend eggs, remove three 100-mL portions of the egg suspension and fertilize these with 1, 3, and 10 mL of the sperm suspension. After 1.5 to 2.5 h, inspect each of these portions microscopically. Use the zygote suspension with the lowest amount of sperm giving normal embryo development (i.e., $\geq 90\%$). Each suspension contains sufficient numbers of embryos to perform multiple tests. The number of tests, however, will depend on the initial density of the egg solution, the test chamber volume, and the final density of embryos in the test chambers.

* Nitex or equivalent.

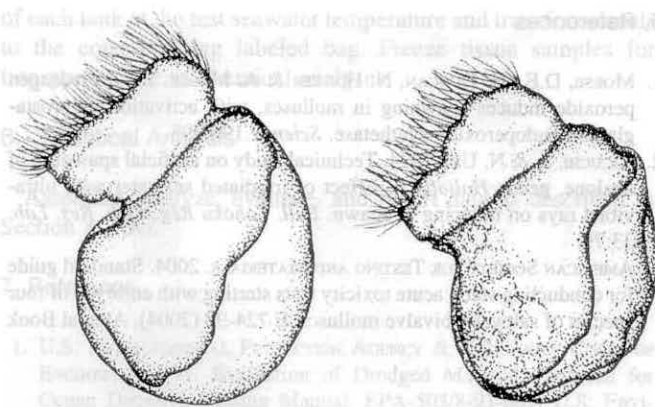


Figure 8610:1. Abalone: (left) normal veliger; (right) abnormal veliger.

b. Setting up test chambers:

1) Marine gastropod larvae—Conduct assay at 15°C and then maintain within $\pm 1^\circ\text{C}$ of that temperature. Maintain photoperiod at 16 h light and 8 h dark; ambient laboratory lighting between 550 and 1050 lux is sufficient. Prepare test chambers as described in Section 8010F. Prepare sufficient water volume to initiate five replicate chambers per test concentration and control with 10 mL of water in each replicate. Measure dissolved oxygen in one replicate from each concentration. Do not aerate chambers unless dissolved oxygen falls below 4.0 mg/L. If aeration is required, do not exceed that necessary to maintain an acceptable minimum oxygen level. Inoculate each test chamber to provide a minimum of 300 embryos from the adjusted stock per 10 mL.

2) Marine bivalve larvae—Conduct assay at $20 \pm 1^\circ\text{C}$ for oysters and clams and $15 \pm 1^\circ\text{C}$ for mussels. For photoperiod, lighting, test chamber preparation, and dissolved oxygen control, see ¶ 1) above. Prepare sufficient water volume for four replicate chambers per dilution and control, with 10 mL water in each. Calculate volume of fertilized egg suspension necessary to provide 15 to 30 larvae/mL and add to each replicate.

c. Performing tests:

1) Marine gastropod larvae—Incubate for 48 h at $15 \pm 1^\circ\text{C}$. At the end of the 48-h period, pour entire test solution with larvae through a 37- μ m-mesh screen if transferring to a container with smaller volume. Wash larvae from screen into 25-mL vials. Add buffered formalin to preserve the larvae in a 5% solution (alternatively, use glutaraldehyde in a 0.5% solution). Pipet a sample from each vial onto a Sedgwick-Rafter counting slide and examine 100 larvae. Return larvae to vials for future reference. Count number of normal and abnormal larvae in each replicate container. Use larval shell development as the test end point (Figure 8610:1). Further details and test variability are available.⁴

2) Marine bivalve larvae—Incubate for 48 h at $20 \pm 1^\circ\text{C}$ for oysters and clams and $15 \pm 1^\circ\text{C}$ for mussels. After incubation period, proceed as directed in ¶ 1) above. Further details and test variability are available.^{3,5}

5. Statistical Analysis

Calculate and report results in accordance with Section 8010G. Computer methods for data processing and analysis are available.³⁻⁵

6. References

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8610 D. Sediment Test Procedures Using Marine Bivalves

1. General Procedures

Before conducting a definitive sediment test with the bivalve *Macoma nasuta*, conduct preliminary tests to become familiar with the test procedures given below.

2. Water Supply

Use seawater with a salinity of approximately 35 g/kg and a pH of about 7.8. A minimum salinity of 25 g/kg is required for the test but any salinity within the range 25 to 35 that reflects site-specific conditions is appropriate. Filter seawater to 20 μ m or less in quantities sufficient to support this testing scheme for its duration. Routinely analyze and document source seawater for metals, especially heavy metals, and other potential contaminants.

3. Sediment

Collect sediment from the site with a benthic sampling device (see Section 10500B). Multiple samples may be required from each individual site to acquire sufficient material. Collect control sediment at the same time and in the same general location as the test organisms. Collect reference sediment from a site known to be generally free of contaminants. Sieve test, control, and reference sediments through a 1.0-mm screen to remove resident organisms. Store sediment at 4°C until required for test initiation; recommended holding times for sediment range from 2 to 6 weeks.¹ Begin tests within 6 weeks of sediment collection.

4. Exposure Chambers

Use 20- to 40-L aquaria for long-term flow-through or static-renewal exposures of *Macoma*; these will generate sufficient quantities of tissue for the chemical analyses necessary to determine bioaccumulated tissue concentrations of virtually any analyte. The duration of bioaccumulation tests (30 d including a depuration period of 2 d) requires the use of relatively large sediment volumes. A 40-L aquarium should contain a 5- to 8-cm depth of sediment with overlying water reaching 4 to 6 cm below aquarium height. Maintain ratio of sediment to seawater for smaller aquaria to ensure that there is enough material to support

the animals throughout the 28-d exposure and 2-d depuration period. Approximately 50 g wet weight sediment per gram of wet flesh (without shell) is an appropriate minimum; however, consider the final volume of tissue needed for the analytes of concern when choosing the number of animals per replicate and ultimately the amount of sediment and water required to support them. Preferably use flow-through facilities if they are available; the clean seawater should flow over the surface of the sediment at the bottom of the tanks at a rate of at least 1 drop/s. If static-renewal procedures are used, siphon 80% of the overlying water in each aquarium and replace with clean seawater every other day at minimum. Cover aquaria to prevent introduction of foreign materials.

5. Conducting the Toxicity Tests

a. Setting up test chambers: Conduct assay at a chosen temperature between 12°C and 16°C and maintain within $\pm 2^\circ\text{C}$ of that temperature. Although photoperiod does not appear to be a critical parameter for sediment testing with clams, maintain and report a 16:8, 14:10, or 12:12 light:dark period. Ambient laboratory lighting is sufficient. Prepare test chambers as described in Section 8010F. Prepare sufficient aquaria to initiate five replicate chambers per site, as well as five replicates each for control and reference sediments. Each site is an individual test material; there is no dilution series or sediment concentration associated with bioaccumulation testing with clams (although such a design is possible). Label each chamber with an identifier. Place an appropriate volume of sediment (as described in C.3) at bottom of each test chamber. A total volume of about 60 L sediment per site (before sieving) is sufficient for testing in 40-L aquaria. Adjust volume to reflect test chamber size and number of animals used per replicate. Choose a test temperature between 12°C and 16°C, but maintain within $\pm 2^\circ\text{C}$ of the chosen temperature.

b. Performing tests: Set up test chambers as described above. After equilibration, add mollusks to each tank. Thirty clams will be well supported by 5 to 8 cm of sediment in a 40-L aquarium with overlying seawater. This number of animals is sufficient to ensure adequate tissue for the chemical analyses typically associated with this type of testing. This assay is often used to expose more than one organism type simultaneously. Should additional animals be included in the analysis, ensure adequate sediment

and water for their health. After adding animals, record initial measurements of temperature, dissolved oxygen, pH, and salinity, and confirm that values are within the appropriate range for testing. Remove a water sample from each tank and hold for ammonia analysis. The duration of exposure for determining bioaccumulation is 28 d with an additional 2-d exposure to clean sediment for tissue depuration. See Section 8610B.2b2) for organism information.

Examine each replicate daily and monitor for mortality. Remove dead organisms immediately to preserve water quality. All dead clams will not necessarily be accounted for because of tissue degradation and scavenging by remaining clams. Note removal of dead organisms on water quality observation sheet. Record daily monitoring of salinity, dissolved oxygen, pH, and temperature on a water quality data sheet. On the day of initiation, every fifth day during the test period, and at test termination, remove a subsample from one replicate of each sediment type (i.e., site, control, or reference) for ammonia analysis. Adjust flow rate of the water if dissolved oxygen falls below 4 mg/L.

On Day 28 of the assay, transfer the animals to clean seawater to depurate for 48 h. Label equivalent size test chambers with the same identifiers and place control sediment in the bottom to the same level as test sediment. Sieve contents of each test chamber with clean seawater at the test temperature and transfer surviving organisms into control sediment. In preparation for test termination, label food-grade resealable plastic bags with species name and replicate identifiers. After depuration period, sieve contents

of each tank at the test seawater temperature and transfer animals to the corresponding labeled bag. Freeze tissue samples for transport to site of chemical analyses.

6. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

7. Reference

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8610 E. Field Test Procedures Using Freshwater and Marine Bivalves

1. General Procedures

Conduct controlled experiments with caged bivalves under field conditions to characterize chemical exposure and associated biological effects in the same organism. Obtain test specimens, introduce them into cages, and place in areas of concern. Exposure can be characterized by measuring the accumulation of chemicals in tissues or by measuring biochemical changes. Effects can be assessed by measuring end points such as survival, growth, and reproduction.

Although it is possible to measure chemicals in individual bivalves, this method assumes that, for cost-effectiveness, tissues will be pooled for chemical analyses. The number of individuals necessary for the composite depends on amount of tissue required for analysis.

2. Water Supply

The water where bivalves are transplanted should be sufficient for growth or, at a minimum, maintenance of tissue mass, so that available chemicals will be accumulated in their tissues. Consult published information¹ on normal temperature and salinity ranges for commonly transplanted marine and freshwater species.

3. Exposure Chambers

a. Types of cages: Although it is possible to mark individuals, cages with individual compartments are recommended for field studies with caged bivalves to minimize interferences, standardize procedures, and minimize handling individuals. The cage is designed to maximize water flow to the test animals while containing them within the cage to ensure that each individual has the same water exposure. Cages can be flexible material with compartments attached to a rigid frame (i.e., mesh bags attached to a PVC frame; Figure 8610:2) or rigid with fixed compartments (i.e., plastic trays or wire baskets with internal divisions). Cages without compartments can be used, but this is not recommended because of the potential for clumping of individuals and uneven exposure conditions. Compartmentalization facilitates tracking individuals throughout the test and eliminates the need to mark them. Although there are techniques for numbering individuals,^{2,3} this approach is time-consuming if large numbers of animals are used. Using glues or epoxies for marking may introduce potentially toxic chemicals.

Rigid cages with fixed compartments have been used in freshwater^{4,5} and marine⁶ environments. PVC frames supporting flexible mesh material have been used in freshwater, estuarine, and marine habitats.^{7,8} Different cage designs, including rigid cages with and without compartments, corrals that limit the movement

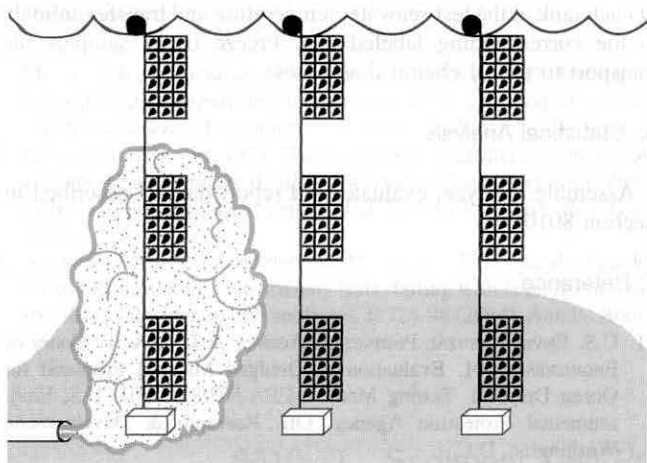


Figure 8610:2. Schematic layout of cages, consisting of mesh bags attached to PVC frames, suspended from a line attached to a buoy at water surface and anchor at bottom.

of sediment-dwelling bivalves, and leashes where monofilament lines were glued to each bivalve shell, have been compared with natural conditions.⁹

b. Flexible mesh and PVC cage construction: The final dimensions of the PVC frames and the mesh bags depend on the species, size of organism at the start of the test, expected growth, and the number of organisms per cage. Oyster culch netting, used in bivalve aquaculture, is recommended material for the mesh bags; tubular oyster culch netting is available in many diameters and mesh sizes. A 15-cm-diam mesh material is recommended for smaller smoothed-shelled species, such as mussels and clams, because there is less excess mesh at the point of constriction. Use tubing of larger diameter for larger, rough, and irregular shells such as oysters. Create individual compartments by separating the bivalves within the mesh bags with a plastic cable tie. Provide sufficient space in each compartment for test animal growth during the test. The mesh bag should be of sufficient length to accommodate the desired number of bivalves per bag plus sufficient material for attachment to the frame. Approximately 60 cm of mesh netting is sufficient for attachment to a PVC frame measuring 1.9 cm material in diameter. Frames can be constructed from aluminum, stainless steel, or plexiglass, but PVC pipe (1.25 or 1.9 cm in diameter) is preferred. Frame sizes range from 30 × 30 cm to about 22 × 100 cm. To minimize crowding and possible damage to shells, choose a frame width approximately 5 cm longer and wider than the space occupied by the bivalves after they have been placed in the bag. Soak frames in flowing fresh or seawater for at least 24 h after construction, to remove any volatile chemicals.

Drill holes about 0.6 cm in diam at intervals of about 24 cm to allow water to enter the pipe and remove trapped air, to obtain neutral buoyancy and prevent floating of the frame. To maintain frame strength do not drill at the corners.

Construct compartmentalized cages for holding the bivalves from high-density polyethylene, polycarbonate, or fluorocarbon plastic, or from glass, Type 316 stainless steel (fresh water only), or nylon, to minimize dissolution, leaching, and sorption. Do not use plastic cable ties containing metal stops, since corrosion may occur.

4. Conducting the Tests

a. Preparation for distribution: Label mesh bags with identification tags made of durable plastic or other inert material. Attach tags to bags with plastic cable ties. Preferably knot bag approximately 30 cm from the end and attach tag near knot. Indicate both cage number and bag number on tag. Colored beads can be used as a backup labeling system to identify bag number on the PVC frame in the event it becomes dislodged.

Separate bags into groups according to bag number because the distribution is based on bag number with all bags of a common number filled at a given time. For example, in a study with ten cages and each cage holding three bags of bivalves, the following sequence of labels would be prepared, where the first number indicates cage number and the second bag number:

1-1, 2-1, 3-1, 4-1, 5-1, 6-1, 7-1, 8-1, 9-1, 10-1 (these are all the Bag 1 for the 10 cages)

1-2, 2-2, 3-2, 4-2, 5-2, 6-2, 7-2, 8-2, 9-2, 10-2 (these are all the Bag 2 for the 10 cages)

1-3, 2-3, 3-3, 4-3, 5-3, 6-3, 7-3, 8-3, 9-3, 10-3 (these are all the Bag 3 for the 10 cages)

After labeling, gather all bags with the same bag number designation; there should be one for each cage number. Fill bags in groups, starting with the -1 group first. Stretch the mesh material by hand to approximately 6 × 6 × 12 cm so that the bivalve falls into the bag. Attach bags to frame (as if hanging stockings on a clothes line) starting with the -1 bags, so that the bags are in cage number sequence.¹

b. Presorting: To minimize size differences among cages at the start of the test, presort bivalves into size groups. Do not use specimens with holes or broken shells. Base sorting on either shell length, as determined with vernier calipers, or whole-animal wet-weight. After presorting, determine the number of bivalves in each size category. Initiate test using the minimum size range with a target range of 5 to 10 mm (i.e., 20 to 25 mm, 33 to 38 mm, 36 to 46 mm) that contains the maximum number of individuals. Keep animals moist and cool during presorting, as described below. Keep all bivalves originally collected until the test is initiated to ensure there is a sufficient number of individuals.

Handle specimens as carefully, gently, and quickly as possible to minimize stress. When bivalves are transported a long distance, do not keep them submerged in water, but keep them moist and cool by placing them in an ice chest with either wet ice or frozen gel packs on the bottom; place newspaper, paper toweling, or cloth toweling between specimens and ice to prevent direct contact. Wet towels also can be placed over the specimens to provide additional moisture.

Keep organisms out of water before the test to eliminate exposure to oxygen-deficient conditions unless specimens are placed in a nonrecirculating flow-through system. Do not expose specimens to direct sunlight and keep them moist as described above. If the air temperature is high, keep specimens in an ice chest with wet ice as described above.

c. Preparing bivalves for distribution to mesh bags: Once the size range has been determined, make more precise shell length and/or whole-animal wet-weight measurements. It may not be possible to measure shell length in irregularly shaped specimens, such as oysters. Place all bivalves within the size group in a tray containing water. Maintain specimens in water during the mea-

surement and distribution process to eliminate entrapment of air between the valves. Monitor water temperature and keep it within approximately $\pm 5^{\circ}\text{C}$ of field temperatures. Plastic bags containing wet ice may be placed in the tub with the bivalves to maintain desired water temperature. A rapid change in temperature may induce spawning of gametes in mature animals, which would add another variable to the test. Keep bivalves completely submerged and flat on the bottom before measurement. Bivalves that float or that are positioned upright on the bottom may have air trapped between their valves; do not use these individuals because the air will bias the weight measurement. Transfer these individuals to a separate container where they may purge the trapped air within 5 to 10 minutes; if this occurs, these animals can be used.

d. Measuring and distribution to mesh bags: Once the bivalves have been placed in a tray filled with water, the specimens will begin to respire and their shells will open slightly (~ 1 mm). Most species close their shells upon physical contact. If any specimens do not completely close their shells upon movement, physical contact, or water agitation, do not use them. Randomly select one specimen from tray, blot excess water from surface, and measure its length and weight. Record data electronically and/or manually. Place specimen into the first mesh bag on the distribution rack. Affix a 10-cm cable tie around mesh material above this specimen, and adjust so that it is tight enough to prevent the animal from passing through but loose enough to be removed if necessary. Do not constrict the mesh to the point that it might restrict valves from opening or growing. Randomly select a second specimen from the tray and repeat the process until each mesh bag contains desired number of specimens. When all bags on distribution rack have been filled, remove bags, knot or cable-tie the open end, leaving a tail length of approximately 30 cm. Place completed bags in a cooler lined with ice and moist paper towels. Repeat the above process until all mesh bags are filled. This process ensures that each cage will have approximately the same number of individuals from each size group.

Once the bivalves have been distributed to all mesh bags, sort bags by cage number (i.e., the first number on the label). Either attach the bags to the PVC frames, as described below, or place them, secured together with a large plastic cable tie, into ice chests for transporting to a holding area in the laboratory or field with uncontaminated flowing water. Hold in water overnight or until ready to attach bags to the PVC cages.

e. Measuring and distributing bivalves to be used for baseline (beginning-of-test) tissue chemistry: During the measurement and distribution of test specimens, identify bivalves to be used for baseline tissue chemistry, to ensure similar sizes. This can be accomplished by randomly assigning a cage number for each baseline tissue chemistry sample. However, the bivalves can be distributed to compartmentalized boxes instead of mesh bags. The use of compartmentalized boxes eliminates the need to remove individuals from the mesh bags. Place the individuals into the compartmentalized boxes in numerical order. At the end of the distribution process, remove the tissues for chemical analysis according to the procedures in ¶ k below.

f. Attachment of mesh bags to PVC frame: A set of mesh bags attached to a PVC frame constitutes a cage (Figure 8610:2). To attach the mesh bags to the frame, knot tail ends of mesh directly to the PVC or use cable ties. Allow sufficient slack in the mesh bag after it is attached for the bivalves to open properly, but not

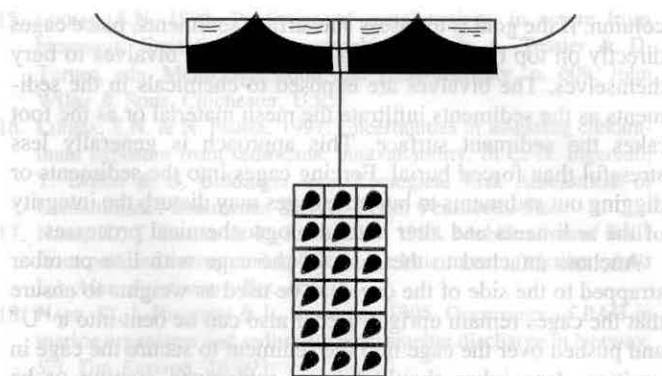


Figure 8610:3. Cage suspended from a fixed mooring.

so much slack that the bivalves are suspended below the plane of the cage. If the mesh is stretched too tightly it will restrict valve opening, filtration, and growth. If the bag is too loose, it will tend to become tangled in the deployment array. If necessary, slide the cable ties to increase or decrease the space between individuals to insure adequate space for respiration and growth. A temperature recording device may be attached to the frame at this time.

g. Water column suspension: Cages can be suspended in the water column either from a fixed mooring (Figure 8610:3), such as a floating pier or piling, or from a line with an anchor on one end and a buoy attached to the other end (Figure 8610:2). Factors to consider during the placement of cages in the water column include tidal fluctuations, bottom topography, and boating and recreational activity in the vicinity. Water-column deployments are used primarily to assess chemicals in the water column. However, if the cage is suspended near the bottom, the test also can be used to assess chemicals associated with sediments.

h. Fixed bottom deployment: Cages can be placed directly on the sediments or set at a fixed distance from the bottom by attaching legs to the cage and pushing the legs into the sediments to hold the cage in place (Figure 8610:4). Bivalves of certain species, caged and placed directly on the sediments, can be used to assess chemicals associated with both sediments and the water

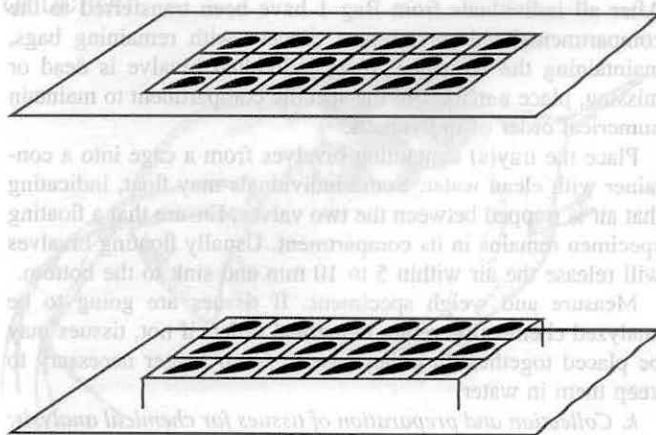


Figure 8610:4. Cages placed (above) directly on sediment and (below) on attached legs a fixed distance above sediment.

column. If the goal is to assess subsurface sediments, place cages directly on top of the sediments and allow the bivalves to bury themselves. The bivalves are exposed to chemicals in the sediments as the sediments infiltrate the mesh material or as the foot rakes the sediment surface. This approach is generally less stressful than forced burial. Forcing cages into the sediments or digging out sediments to bury the cages may disturb the integrity of the sediments and alter natural biogeochemical processes.

Anchor attached to the sides of the cage with line or rebar strapped to the side of the cage can be used as weights to ensure that the cages remain upright. Rebar also can be bent into a "U" and pushed over the cage into the sediment to secure the cage in position. Iron rebar should have a rubberized coating or be covered by plastic bags to prevent water exposure to the metal.

When placing cages on sediments, consider presence of natural vegetation, type of substrate, boating traffic, and recreational activity. More detail on deployment hardware and configurations is provided elsewhere.^{1,10,11}

i. *Deployment period*: Preferably let test run for 60 to 90 d; 30 d is the recommended minimum. If the chemicals of concern are low-molecular-weight organic compounds, such as some PAHs, then a shorter test period can be used. Equilibrium for most chemicals, such as metals or high-molecular-weight organic compounds, usually is achieved in bivalves within approximately 60 to 90 d.^{8,12-20}

j. *Retrieval and end-of-test measurements*: After retrieving the caged bivalves, remove any foreign material, if present, by dipping the cage into the water. Remove any growth from the exterior of the shells. If the bags are removed from the PVC frames, use a separate ice chest lined with wet ice and moist paper towels to transport the bivalves to the laboratory. Cover top and bottom of cages with a tarp or other protective covering to reduce evaporation and prevent temperature elevation and contamination.

Process bivalves from all bags constituting a cage as a unit; retain numerical order so that initial and terminal measurements of each specimen are recorded. Remove the bivalves from the mesh bags and place them in a compartmentalized tray to facilitate end-of-test measurements, starting with Bag 1 and ending with Bag N. Begin at end of the bag with plastic label, since this end was filled first. Remove bivalves and place them, in sequential order, into a compartmentalized plastic tray filled with water. After all individuals from Bag 1 have been transferred to the compartmentalized tray, repeat process with remaining bags, maintaining the sequence. If an individual bivalve is dead or missing, place a marker in the specific compartment to maintain numerical order of individuals.

Place the tray(s) containing bivalves from a cage into a container with clean water. Some individuals may float, indicating that air is trapped between the two valves. Ensure that a floating specimen remains in its compartment. Usually floating bivalves will release the air within 5 to 10 min and sink to the bottom.

Measure and weigh specimens. If tissues are going to be analyzed chemically, retain numerical order; if not, tissues may be placed together. In either case, it is no longer necessary to keep them in water.

k. *Collection and preparation of tissues for chemical analysis*: Shucking knives, cutting boards, trays, and weighing pans are needed for tissue preparation. Use tools made of non-corrosive material such as resistant stainless steel, anodized aluminum, or

borosilicate glass. Before each use wash equipment with a soap-free cleaning solution, rinse in hot tap water, and rinse with deionized water. An acetone or 95% ethanol rinse can follow the last water rinse if required by the analytical laboratory. Let equipment air-dry to prevent contaminating tissues with water. If gloves are worn, keep them powder-free and replace after processing a cage of mussels. If gloves are not worn, wash hands each time in a soap-free cleaning solution.

Retain order of specimens during tissue extraction; record tissue weights separately. To remove tissue, slide a thin knife blade between the two shells and sever the anterior and posterior adductor muscles. (CAUTION: *Danger of injury*.) It may be necessary to notch a clam or oyster shell so that the knife can be inserted. Drain excess water from the animal. Spread the two valves, exposing tissue. Remove tissue with knife blade and place on holding tray.

Weigh tissues individually, using a clean weighing pan each time. Tare between each weight measurement. Place tissues within a cage group in a sample jar, seal, and store according to laboratory specifications. For procedures for analyzing tissues, see Part 3000 (metals) and Part 6000 (organic compounds).

5. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

6. References

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8710 ARTHROPODS

Phylum Arthropoda is the largest group of animals; it comprises more than one million species, the majority of which are insects. Other arthropods include crustaceans, spiders, ticks, mites, and other less-known species. Arthropods are found in all environments,

including both fresh and marine waters. Two classes of arthropods are used extensively in toxicity testing, the crustaceans and the insects. Test procedures are described for several different crustacean groups including *Daphnia* (8711), *Ceriodaphnia* (8712), mysids (8714), and decapods (8740). Representatives of the insect orders belonging to stoneflies, mayflies, caddisflies, and dipterans are the most commonly used groups in aquatic testing (8750).

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8711 DAPHNIA*

8711 A. Introduction

Daphnia sp. (Figure 8711:1) are small freshwater crustaceans. They have been used for many years to assess the acute and chronic effects of single chemicals and complex mixtures.¹⁻³

Daphnia are valuable as test organisms because of their sensitivity to toxic substances, ease of identification and handling, ubiquitous distribution, and extensive use in toxicity testing. *Daphnia* are fecund and reproduce parthenogenically, which allows for the establishment of clones with little genetic variability and with reproducible testing results.

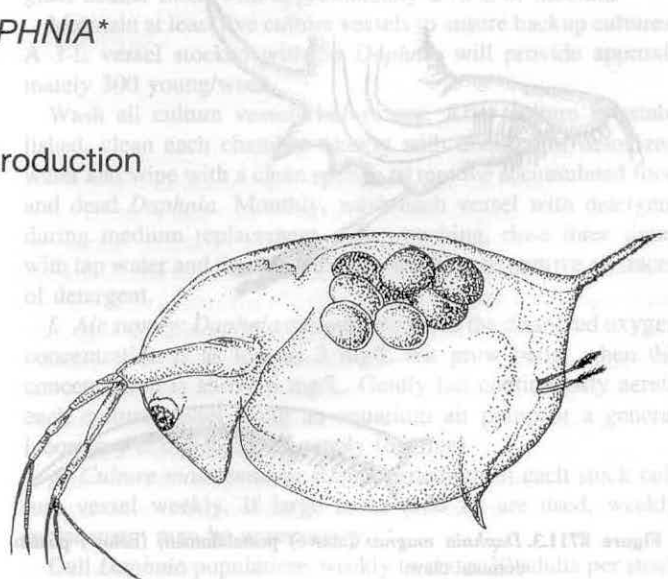


Figure 8711:1. *Daphnia* sp., adult female.

* Approved by Standard Methods Committee, 1997.

Joint Task Group: 20th Edition—Wayne G. Landis (chair), Colin E. Coggan, Joseph W. Gorsuch, Robert E. Morcock, Mark A. Palmieri.