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Office of Water
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NPDES Compliance Monitoring Inspector Training

Biomonitoring

NPDES COMPLIANCE MONITORING INSPECTOR TRAINING MODULE

BIOMONITORING

U.S. ENVIRONMENTAL PROTECTION AGENCY

ENFORCEMENT DIVISION
OFFICE OF WATER ENFORCEMENT AND PERMITS
ENFORCEMENT SUPPORT BRANCH

JUNE 1990

DISCLAIMER

This module has been reviewed by the U.S. Environmental Protection Agency's (EPA's) Office of Water Enforcement and Permits and approved for publication. This module represents EPA's introductory training on selected topics relating to conducting NPDES compliance inspections. Failure on the part of any duly authorized official, inspector, or agent to comply with its contents will not be a defense in any enforcement action, nor will failure to comply with this guidance alone constitute grounds for rendering evidence obtained thereby inadmissible in a court of law. The mention of trade names, commercial products, or organizations does not imply endorsement by the U.S. Government.

AKNOWLEDGMENTS

This document represents an update of an earlier module originally developed by the Enforcement Division of the Office of Water Enforcement and Permits. The module was revised under the direction of Virginia Lathrop and Gary Polvi of the Office of Water Enforcement and Permits, with the review and comment of Shiela Frace of the Office of Enforcement and Compliance Monitoring. In addition, the EPA Regions provided extensive reviews. Many valuable comments were provided, ,most of which have been incorporated into this module. Science Applications International Corporation prepared this updated moduile under EPA Contract No. 68-C8-0066, WA No. C-1-2 (E).

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FOREWORD

This document is one of five training modules developed by the Office of Water Enforcement and Permits, U.S. Environmental Protection Agency (EPA) to introduce the National Pollutant Discharge Elimination System (NPDES) program to new inspectors. Information in each module provides training to an inspector unfamiliar with the NPDES program. The modules address the following topics:

- The Overview presents an overview of the entire NPDES program and briefly summarizes different types of inspections conducted under this program
- Legal Issues discusses the legal issues which must be addressed during an inspection and provides legal information to assist inspectors in performing their duties
- Biomonitoring outlines the principles of biomonitoring and the role of biological testing in the inspection program
- Sampling Procedures details procedures to be used when conducting a sampling inspection
- Laboratory Analysis outlines procedures and information necessary to perform an effective evaluation of a permittee's laboratory.

The modules are best used in a classroom setting where there is discussion between instructors and students and where questions can be asked. Yet, they can also stand alone as reference sources. A more detailed discussion of the topics covered in these modules appears in EPA's 1988 NPDES Compliance Inspection Manual.

These training modules were developed primarily for inhouse training of Regional and State NPDES Inspectors. However, they are available as well to other interested parties such as attorneys, other program offices, facility owners and operators, and members of the general public.

Regional and State personnel are encouraged to provide EPA Headquarters with changes or information which instructors or managers believe would improve these modules. The content of the modules will be updated and revised periodically. Comments, information, and suggestions to improve the modules should be addressed to:

Enforcement Support Branch (EN-338)
Office of Water Enforcement and Permits
U.S. Environmental Protection Agency
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Washington, DC 20460

1. INTRODUCTION

1.1 OVERVIEW OF THE NPDES PROGRAM

The Federal Water Pollution Control Act of 1972, as amended by the Clean Water Act (CWA) of 1977, specifies the objectives of restoring and maintaining the chemical, physical, and biological quality of the Nation's waters. The Act provides broad authority to the U.S. Environmental Protection Agency (EPA) to undertake several activities:

- Establish the National Pollutant Discharge Elimination System (NPDES) program and the National Pretreatment Program
- Define pollution control techniques and establish effluent limitations based on these technologies or the level necessary to protect State water quality standards (whichever is more stringent)
- Obtain information through reporting and compliance inspections
- Take enforcement actions, both civil and criminal, when violations of the Act occur.

The Act also provides authority for the States to undertake activities to protect their waters:

- Establish State water quality standards to protect designated uses
- Apply for authority to run the NPDES program and the National Pretreatment Program.

The NPDES program, mandated by Section 402 of the Act, regulates the discharge of pollutants from point sources such as municipal treatment plants, industries, animal feedlots, aquatic animal production facilities and mining operations. In order to discharge, each point source is required to obtain a NPDES permit containing effluent limits, a compliance schedule, monitoring

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and reporting requirements, and any other terms and conditions necessary to protect water quality.

To determine whether these NPDES permit conditions are being met, Section 308 of the Act authorizes inspections and two types of monitoring of permittee facilities: self-monitoring conducted by the permittee and compliance monitoring conducted by the permit-issuing agency. According to the Act, an inspection may be conducted where there is an existing NPDES permit or where a discharge exists or is likely to exist and no permit has been issued.

Historically, EPA has relied on technology-based standards to control the discharges of point sources, particularly industries. Technology-based standards for industrial point sources are targeted on regulated pollutants, known as conventional, nonconventional, and toxic pollutants, and on the industrial categories that contribute the majority of regulated pollutants.

Technology-based limits on conventional pollutants, the typical emphasis of many early NPDES permits, has resulted in improved receiving water quality. However, the CWA also requires that permit limits reflect State water quality standards in the form of numeric standards on individual toxicants and narrative standards prohibiting toxic conditions in general. For this reason, permits may require additional limits on specific toxicants or whole effluent toxicity (WET).

Whole effluent toxicity limits are designed to measure the toxic impact of the effluent on biota and are a necessary addition to chemical-specific limits for several reasons:

- The interactions of individual constituents of wastewaters are greater than predicted by summing the effects of individual toxicants
- The toxic effects of many compounds are unknown

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- The bioavailability of many compounds changes with changes in pH, temperature, or even the presence of oxygen.

1.2 CONCEPT OF TOXICITY

By definition, toxicity is a characteristic of a substance (or group of substances) that causes adverse effects in organisms. Adverse effects are mortality and those effects that limit an organism's ability to survive in nature. Toxicity of a substance is measured by observing the responses of organisms to increasing concentrations of that substance. One substance is more toxic than another when it causes the same adverse effect at a lower concentration.

For any given substance, toxic effects are alleviated when the concentrations to which organisms are exposed are reduced. Thus, by reducing the toxicity of a discharge (by reducing the concentrations of toxic constituents), the toxic effect of that discharge on receiving waters is also reduced. Similarly, greater dilution of a toxic discharge will lead to lower toxic effects in receiving waters. The key to effective toxics control is the limitation of measured toxicity in a discharge.

Toxicity testing is the focus of this training module. However, this module is only a brief introduction to toxicity testing and should not be used as a reference for testing procedures. Check with the Regional biologist for advice and recent changes in published toxicity testing procedures.

1.3 TOXICITY TESTING IN THE NPDES COMPLIANCE MONITORING PROGRAM

Toxicity testing is either performed or evaluated in five NPDES inspections:

- Compliance Evaluation Inspection (CEI)
- Compliance Sampling Inspection (CSI)

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- Performance Audit Inspection (PAI)
- Toxics Sampling Inspection (XSI)
- Compliance Biomonitoring Inspections (CBI).

Depending on the inspection, toxicity testing accomplishes one or more of the following six purposes:

- Determines compliance with State water quality standards
- Determines compliance with permit conditions
- Evaluates quality of self-monitoring data
- Assesses self-monitoring performance
- Examines facilities, equipment, records, and reports for self-monitoring
- Develops permit limits.

This module is designed to introduce new inspectors to the concepts and practices of toxicity testing as it relates to CBIs. It is not intended to be a definitive source of material on toxicity testing; rather, its focus is on explaining the points pertinent to CBIs. After mastering the material in this module, an inspector should be able to:

- Define terms used in whole effluent toxicity testing
- Describe whole effluent toxicity testing procedures
- List key aspects of toxicity critical to interpretation of results

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- List guidance documents that can be used to implement CBIs.

Appendix A contains questions and answers that an inspector can use to self-test him/herself after completing this module.

This module provides a basic introduction to toxicity tests. More specific information can be found in the following manuals:

- NPDES Compliance Inspection Manual, May 1988.
- Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.
- Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, EPA/600/4-89/001, March 1989.
- Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters on Marine and Estuarine Organisms, EPA/600/4-87/028, May 1988.
- Technical Support Document for Water Quality-based Toxics Control, EPA-440/4-85-032, September 1985 or more recent revisions.
- Permit Writer's Guide to Water Quality-based Permitting for Toxic Pollutants. EPA 440/4-87-005, July 1987.

Before a CBI is carried out, the inspector is strongly advised to consult one or more of these manuals for further explanation and to consult the Regional biologist.

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2. BASICS OF TOXICITY TESTING

2.1 TOXICITY TEST DESIGN

Toxicity tests are techniques to determine the relative toxicity of a permittee's discharge or effluent by measuring the responses of organisms to solutions containing various percentages of effluent and dilution water. In general, test organisms are exposed to a series of solutions that contain a range of effluent dilutions. However, test designs vary, depending on how the results are to be used. Three general types of tests are performed:

- Range Finding Test - These are usually 24-hour tests conducted to determine the approximate level of toxicity of an effluent, and the concentration to be used in definitive tests. The test organisms are exposed to a wide range of concentrations to determine the highest concentration that killed no (or few) organisms and the lowest concentration that killed all (or most) organisms.

- Screening Test - In this type of test, organisms are exposed to only one dilution of effluent. If a toxic response is observed (see next section), further testing may then be conducted on the effluent. The use of screening tests varies from Region to Region and from State to State. Some Regions and States rely heavily on screening tests to reduce the effort required for detailed testing. Other Regions and States, who feel the benefits of definitive data outweigh the incremental costs, insist that definitive tests be done on effluents requiring toxicity testing.

- Definitive Test - This test estimates the concentrations at which a certain percentage or significant number of organisms exhibit a certain response. In a definitive test, several groups (replicates) of organisms are exposed for a predetermined length of time to solutions of various proportions of effluent and dilution water (Figure 2-1). The response of each organism in each test concentration is observed and recorded, and the number of responses are analyzed in relation to the concentration of effluent to which they were exposed.

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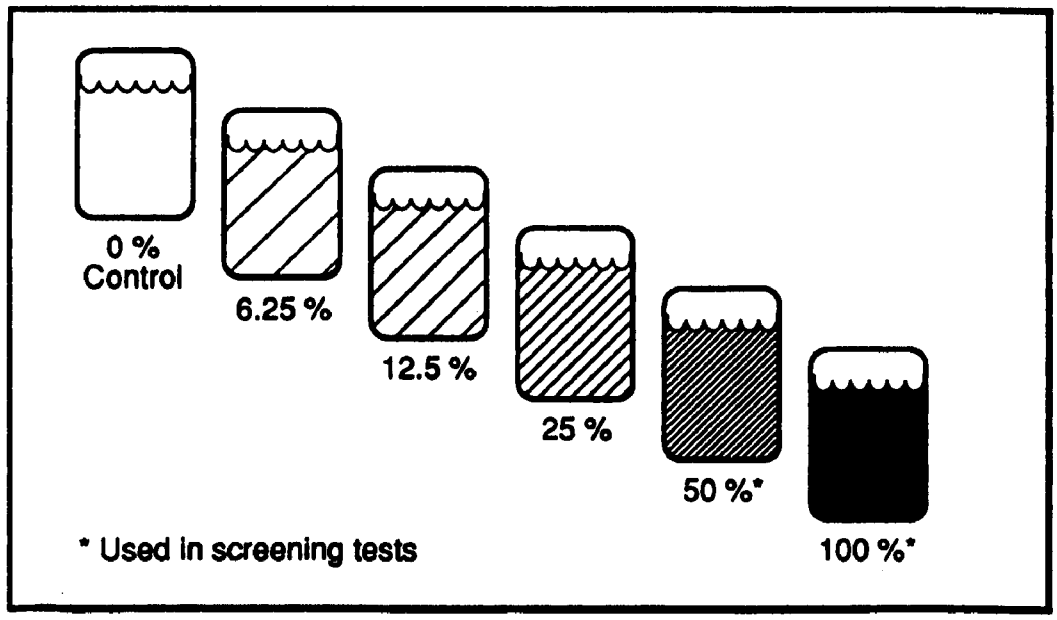
FIGURE 2-1.

TYPICAL EFFLUENT CONCENTRATIONS USED IN TESTING



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Responses that are commonly observed in each test can be one or more of the following:

- Death - Number of organisms killed by a test solution
- Growth - Increase in body weights or sizes of test organisms (e.g., the growth of a female fish is directly proportional to the number and quality of eggs she will produce at maturity)
- Reproduction - Number of offspring produced per female or increase in numbers of organisms
- Terata - Number of gross abnormalities shown in early life stages.

These responses relate directly to an organism's ability to survive in nature and, thus, can indicate whether an effluent will jeopardize the survival of the test species in receiving water.

2.2 ACUTE AND CHRONIC TESTS

Toxicity tests are generally described as either acute or chronic tests. Acute and chronic refer to the length of time organisms are exposed to toxicants before adverse responses are observed. Acute toxicity measures short term effects with impacts usually resulting in death or extreme physiological disorder to organisms immediately or shortly following exposure to a contaminant. Chronic toxicity involves long-term effects of small doses of a contaminant and their cumulative effects over time. These effects may ultimately lead to the death of the organism or disruption of functions such as reproduction or growth.

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2.3 FLOW-THROUGH, STATIC RENEWAL, AND STATIC TESTS

Toxicity tests are also described according to the way in which organisms are exposed to test solutions. In a **flow-through** test, effluent and dilution water are mechanically renewed numerous times a day. This test setup requires specialized equipment (a serial or proportional dilutor or syringe pumps) and is more costly to operate than a static test. In a **static renewal** test, the test solutions are replaced periodically (usually daily) with fresh effluent and dilution water. In a static test, the solutions used at the start of the test are not replaced for the test's duration. Both static renewal and static tests require only basic equipment.

Toxicity tests may be conducted on-site (at the effluent discharge) or off-site (at a central laboratory). The major advantage of on-site testing is the guaranteed exposure of test organisms to volatile or biodegradable toxic constituents. When toxicity may be due to volatile compounds [e.g., chlorine from Publicly Owned Treatment Works (POTWs), which may be lost, on-site testing may be more appropriate. Generally, static renewal and static tests are performed off-site because the cost of maintaining a mobile laboratory on-site is usually more expensive that shipping effluent to a laboratory. Flow-through testing, which requires large volumes of both effluent and dilution water, is generally performed on-site due to the difficulty of obtaining and shipping enough effluent (and dilution water) to continuously replenish the water in test containers.

2.4 ANALYSIS OF TEST RESULTS

With appropriate testing conditions, data generated by definitive toxicity testing are easily interpreted. A sample of typical results appears in the semilogarithmic plot in Figure 2-2. The percentage of test organisms showing a particular response is plotted against the percentage of effluent in the test solutions. This graph is typical in that it shows a generally increasing percent response for increasing percentages of effluent. The graph is also typical in that it shows a change

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FIGURE 2-2.

RESPONSE CURVE FOR A HYPOTHETICAL EFFLUENT CHRONIC TOXICITY TEST

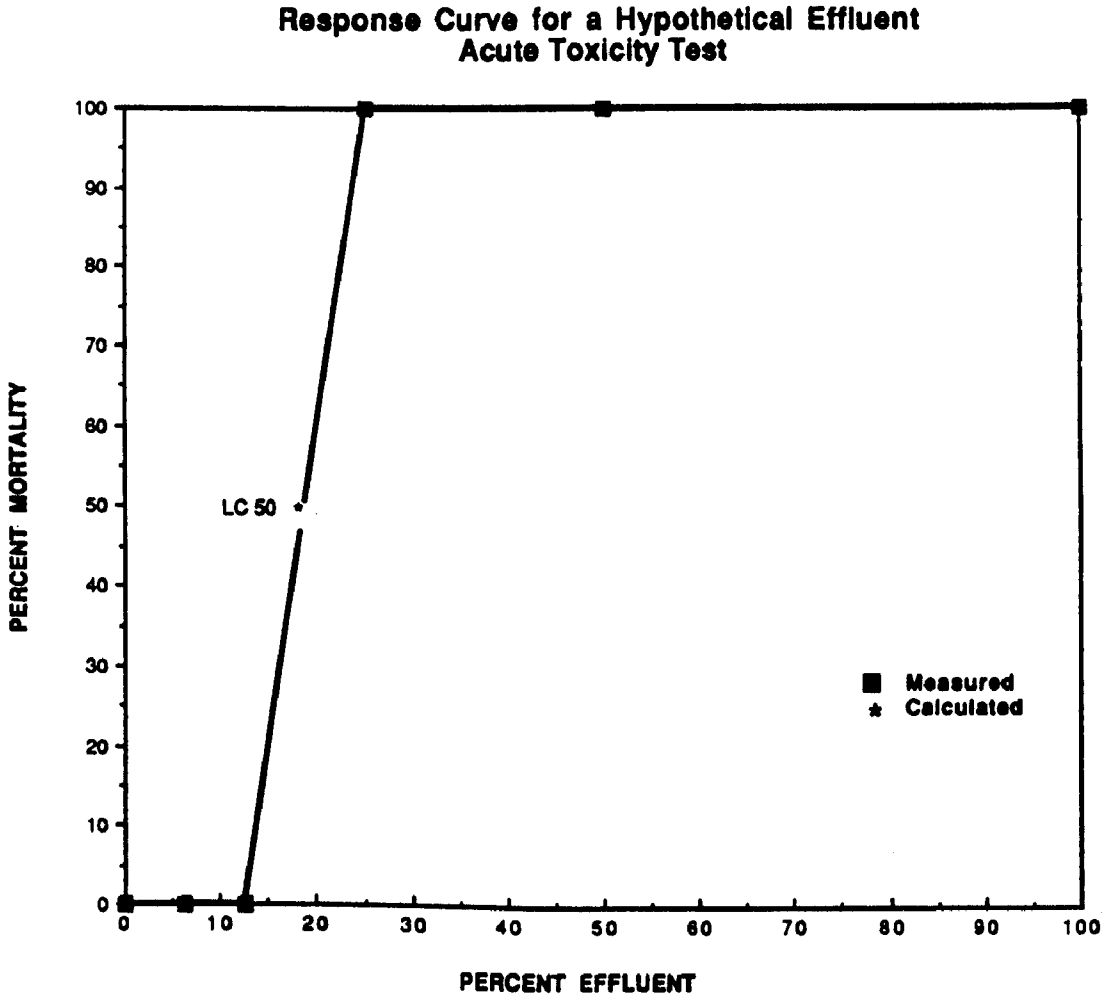

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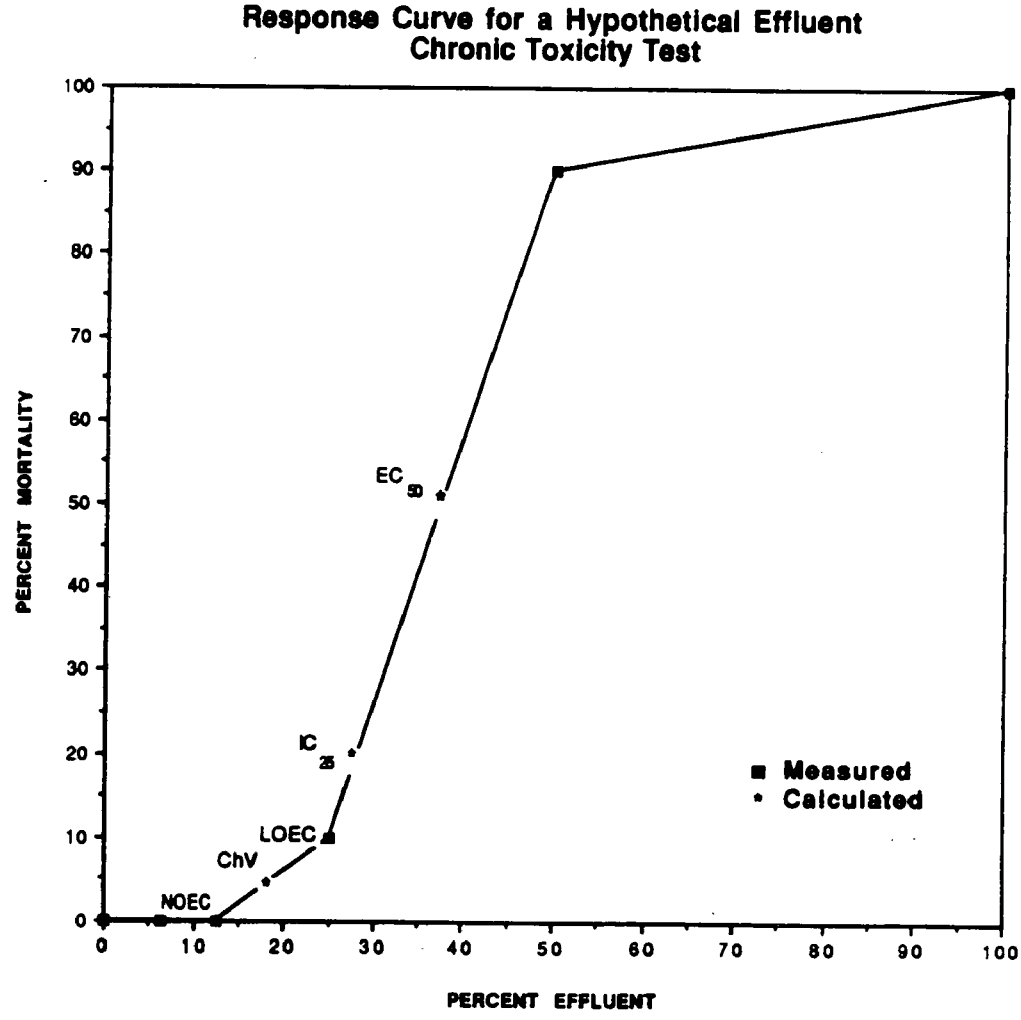
FIGURE 2-3.

RESPONSE CURVE FOR A HYPOTHETICAL EFFLUENT ACUTE TOXICITY TEST



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in response of 75 percent or more between two test concentrations. Sometimes this response is even more exaggerated, with no responses at one concentration and 100 percent responses at the next higher concentration as shown in Figure 2-3.

The terms LC_{50}, EC_{50}, NOEC, LOEC, ChV and IC_{25} refer to different measures of toxicity and are explained below. These various measures of toxicity are also illustrated graphically in Figures 2-2 and 2-3 through the use of a hypothetical example. In these graphs, increasing concentrations of hypothetical effluent produce differing levels of toxic response (as measured by species growth or mortality in these cases).

The LC_{50} (for lethal concentration) is the calculated percentage of effluent (point estimate) at which 50 percent of the organisms die in the test period. In Figure 2-3, the LC_{50} is indicated at the concentration corresponding to 50 percent responses in the test solution. While graphics such as this can be used to determine LC_{50}s, these methods do not allow the calculation of the error associated with LC_{50} estimation. Usually the LC_{50} is calculated statistically by computer programs that fit the response curve to a mathematical function. Computer-based calculation procedures usually print an estimate of the error associated with the LC_{50} estimate.

The EC_{50} is the calculated concentration (point estimate) at which 50 percent of the organisms show a particular effect (not necessarily death). For some species (e.g., *Ceriodaphnia dubia*) where the point of death is not certain, immobility is often used as a surrogate for death. Results for responses like the immobility responses in Daphnia may be reported as an EC_{50} (calculated in the same manner as the LC_{50}). Often, however, no distinction is made between EC_{50}s and LC_{50}s when the response is a surrogate for death.

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The NOEC (no observable effect concentration) is the highest tested concentration at which the organisms' responses are not significantly different statistically from controls. The NOEC (like the LOEC and ChV defined in the following paragraphs) is normally determined only for chronic tests. In Figure 2-2, the NOEC is 12.5 percent effluent since the response in the 12.5 percent effluent is not significantly different from the control response, but the 25 percent effluent response is significantly different from controls.

The LOEC (lowest observable effect concentration) is the lowest tested concentration at which organisms' responses are significantly different statistically from controls. This occurs at 25 percent effluent in Figure 2-2. The ChV (chronic value) is the calculated geometric mean of the NOEC and LOEC (the square root of the product of the NOEC and the LOEC). The ChV is 17.7 percent effluent for the data in Figure 2-2.

The IC_{25} (inhibition concentration) is the calculated percentage of effluent (point estimate) at which the organisms exhibit a 25 percent reduction in a non-quantal biological measurement such as fecundity or growth.

Each of these toxicity test results has units that are concentrations of chemicals or percentages of effluent. As was explained in the Introduction, a substance having an LOEC (or ChV, or any other reporting measure) that is lower than that for another substance is more toxic than the other substance when toxicity is measured using the same test and the same species. For example, one effluent with an EC_{50} of 42 percent effluent is more toxic than an effluent with an EC_{50} of 84 percent effluent.

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There is an inverse relationship between toxicity and the effluent concentration percentage causing a toxic response. In other words, the same toxicity test response (e.g., LC_{50}) at lower percentages of effluent (i.e., more dilution) indicate higher toxicity than test results at higher percentages of effluent using less dilution. A toxic unit, which is directly proportional to toxicity, is sometimes used to express the effluent's toxicity. **Toxic units** are defined as $100/LC_{50}$ for acute or 100/NOEC for chronic when the LC_{50} or NOEC are expressed as percent effluent. An effluent with an LC_{50} of 50 percent effluent has an acute toxicity, TU_a, of 2 acute toxic units. Similarly, an effluent with a NOEC of 25 percent effluent has a chronic toxicity, TU_c, of 4 chronic toxic units. The major advantage of using toxic units to express toxicity test results is that toxic units increase linearly as the toxicity of the effluent increases. So an effluent with a TU_c of 4 is twice as toxic as an effluent with a TU_c of 2. A second advantage to using toxic units is that they are directly analogous to constituent concentrations and can be used in wasteload allocations with the same equations as individual constituents.

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3. TOXICITY TEST COMPONENTS

Reliable toxicity test results can only be expected when each of several key components are handled within the minimum quality assurance requirements discussed below. It is, therefore, very important to understand the relationships between these components and the critical factors that determine the acceptability of each from a quality assurance standpoint. Toxicity tests consist of five components:

- Effluent
- Dilution water
- Test system
- Test organisms
- Test results.

In simple terms (Figure 3-1), effluent and dilution water are combined in the test system with test organisms to produce test results. Each component must be of a specific quality for successful toxicity testing. If the dilution water, for example, is toxic to test organisms, it will be impossible to determine the toxicity caused by the effluent. Similarly, if inappropriate materials are used for the test system, or if the test system is not adequately prepared, observed toxicity might not be due to the effluent, but to the test system itself. Since the objective of toxicity testing is to obtain toxicity data on a given effluent, any factor that could be a source of toxicity, other than the effluent, must be carefully controlled.

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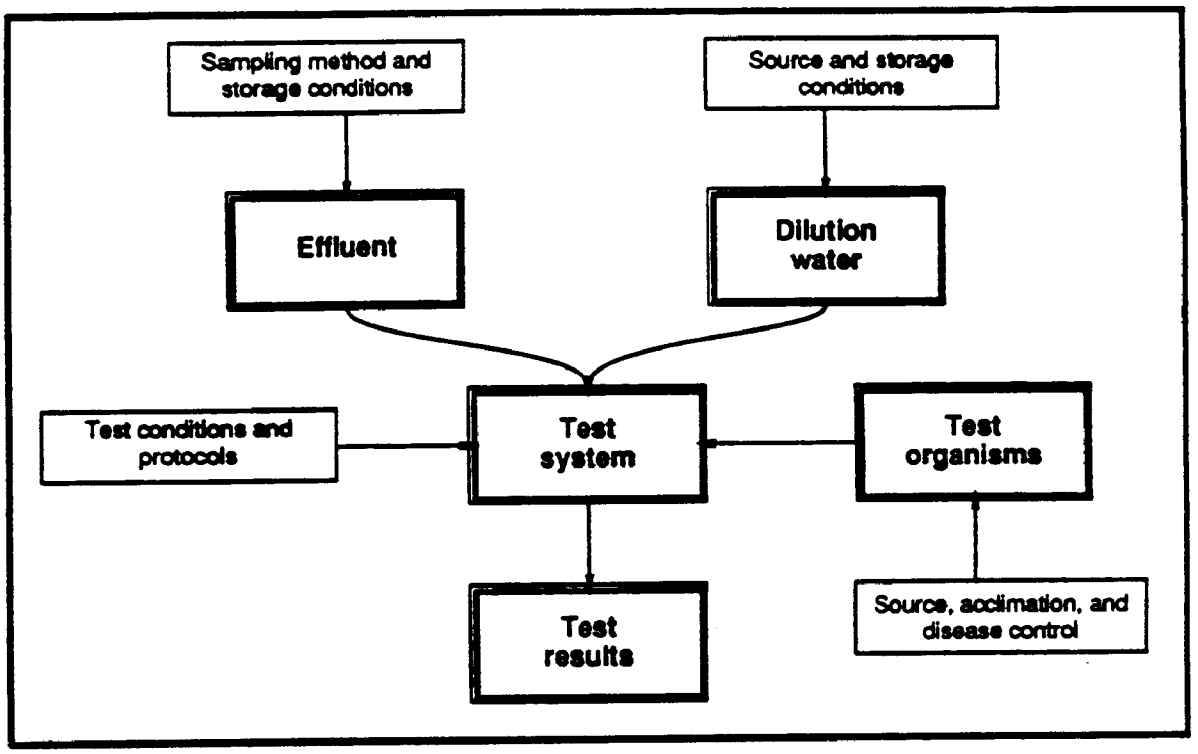
FIGURE 3-1.

RELATIONSHIPS BETWEEN TOXICITY TESTING COMPONENTS, SHOWING IMPORTANT FACTORS FOR EACH



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Factors important for each of these test components are briefly outlined below. Chapters 4-8 of this module then develop each component in more detail. An inspector must keep these considerations in mind whenever evaluating a permittee's toxicity testing procedures. An inspector should also be familiar with other reference sources mentioned in Chapter 1. Appendix B also lists EPA recommended test conditions for the test species most commonly used in aquatic toxicity testing.

When examining a permittee's laboratory facilities, an inspector should also be aware of standard health and safety procedures. A list of standard health and safety precautions is included in Appendix C.

3.1 EFFLUENT

Effluent must be sampled at points specified in the NPDES permit and stored in such a way that the sample is representative of the entire effluent, does not appreciably change in characteristics before it is tested, and is taken without contamination from other sources. An effluent of variable quality may be sampled continuously or as a series of grab samples in order to obtain samples that represent the effluent as much as possible. Single grab samples are usually adequate for unvarying acute tests of effluents. Three or more grab samples are recommended for chronic tests.

Sample containers must prevent the loss of volatile components in order to preserve the representativeness of the sample. Further, the sample must be used in the toxicity test before significant changes occur to any toxic characteristics that may be present. Toxicity can vary because chemicals are lost due to volatilization, chemical precipitation, or biological degradation. Samples should be tested within 36 hours of the time taken. The sample should also usually be refrigerated or stored on ice to prevent biological degradation of organic materials. Sample contamination is avoided by using clean sampling gear and sample containers.

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The inspector should refer to the companion training module on Sampling Procedures for more background information on sampling.

3.2 DILUTION WATER

Dilution water is chosen depending on the objective of the test. Either reconstituted water, receiving water, or other natural waters are appropriate for specific purposes. Dilution water should be specified in the NPDES permit. The type of water appropriate for specific tests is discussed in Chapter 5 of this module.

Dilution water, by itself, should not be toxic. Tests that have significant mortality in dilution water controls should be discarded. If dilution water causes mortality in test organisms during acclimation, the dilution water should not be used in toxicity testing.

3.3 TEST SYSTEM

Materials used to construct the system must prevent contamination of the sample and dilution water, test conditions must be appropriate for the test species, and effluent dilutions used in testing must be accurate. All containers, valves, and tubes of the test system must be of materials that do not provide or remove toxicants and must be thoroughly cleaned before testing. In addition, key variables, such as temperature and dissolved oxygen, must be controlled to ensure test reproducibility.

3.4 TEST ORGANISMS

Test organisms used in toxicity testing must be of known history, free of disease, and acclimated to test conditions. The organisms should also respond "normally" to reference toxicants. "Wild" organisms (i.e., those taken from natural waters) are not generally appropriate for toxicity testing unless cultured in the laboratory for several generations. Most test organisms can be

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obtained from commercial supply houses or laboratory cultures. Diseased organisms should not be used in toxicity testing since the disease will affect the organisms' response to any toxicants in an effluent.

Organisms to be used for toxicity testing should be acclimated slowly to the temperature, salinity, and pH conditions in dilution water prior to the test. These parameters can affect test results substantially; unadapted organisms may exhibit responses to changes in these parameters that may be confused with a toxic response.

A wide variety of organisms can be used for toxicity testing, but acceptable test organisms for compliance monitoring are normally specified in the discharger's permit. The same test organism should be used for the compliance inspection. Two types of freshwater organisms most commonly used in toxicity test inspections are fathead minnows *(Pimephales promelas)* and water fleas (*Ceriodaphnia dubia*). Saltwater species used for toxicity testing include the sheepshead minnow (*Cyprinodon variegatus*), the inland silverside (*Menidia beryllona*), the mysid shrimp (*Mysidopsis bahia*), and the sea urchin (*Arbacia punctulata*).

3.5 TEST RESULTS

Test results must show clear responses to increasing dilutions of effluent, and responses in controls must be negligible. The expected result for toxicity tests is increasing responses with increasing percentages of effluent. If one or more dilutions yield responses inconsistent with this pattern, it is likely that either those dilutions were incorrectly labeled or that contamination occurred in one or more dilutions. The inspector should view such results as suspicious and should require further technical evaluation of the results.

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Organisms in controls (i.e., dilutions with no effluent) should not exhibit any significant number of deaths or other adverse effects. Controls are the basis to determine toxic effects of effluents. If appreciable responses occur in controls, results based on the data will be inaccurate.

3.6 SUMMARY

The quality of the test results is based on proper handling and set-up of the effluents, dilution water, test organisms, and the test system. The results of any particular test indicate the rigor with which these factors were considered during testing. These factors are explained in greater detail in the following four Chapters of this module. If results show questionable patterns or if there are significant responses found in controls, the inspector should not use them to determine a permittee's compliance status.

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4. EFFLUENT

The first major component discussed in detail is the effluent to be tested. The way in which effluents are sampled and stored will determine whether any given toxicity test can be claimed to be representative of the effluent or discharge. Representativeness of the sample may be an issue during any legal proceedings that might arise as a result of an inspection, so adherence to the principles and procedures in the EPA guidance manuals (see Section 1.3) is critical to avoid problems.

Effluent samples must be representative of the discharge. If holding is necessary, the samples must be stored under strict conditions and for limited times so that no appreciable change in toxic characteristics occurs before testing. All samples also should be taken at the location specified in the permit unless the toxicity of particular wastestreams is being evaluated.

4.1 SAMPLING STRATEGIES

The type and frequency of samples taken (e.g., grab, composite) must be consistent with those required in the permit. For flow-through tests that are not done by pumping effluent directly into dilutors, daily sample sizes must be sufficient to supply the dilutor for 24 to 36 hours. This volume will depend on the type of test being conducted and the number of dilutions being run (see Chapter 7 on Test System). For static-renewal and static tests, daily sample volumes should be sufficient to replenish all dilutions in the test series and to provide separate vials of the dilutions to allow for DO, pH, and salinity analysis without contaminating the test dilutions. This volume will depend on the type of test being conducted and the dilutions being run. Refer to Table 4-1 for more information on sampling strategies.

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TABLE 4-1.

RECOMMENDED SAMPLING STRATEGY FOR CONTINUOUS AND INTERMITTANT DISCHARGES FOR FLOW-THROUGH, STATIC RENEWAL, AND STATIC TOXICITY TESTS

Continuous Discharge

Test Type	Preferable	Retention Time ≤14 Days	Retention Time >14 Days
Flow-through	Pump effluent directly to dilutor	Two grab samples daily (early AM and late PM)	One grab sample daily
Static renewal		Four separate grab samples are taken each day for four concurrent tests	One grab sample daily
Static		Four separate grab samples are taken on first day for four concurrent tests	One grab sample on first day

Intermittent Discharge

Test Type	Continuous Discharge During 1 or 2 Adjacent 8-hour Shifts	Discharge from Batch Treatment	Discharge to Estuary on Outgoing Tide
Flow-through	One grab sample midway through shifts	One grab sample of discharge daily	One grab sample of discharge daily
Static renewal	One grab sample midway through shifts daily	One grab sample of discharge daily	One grab sample of discharge daily
Static	One grab sample midway through shifts on first day	One grab sample of discharge on first day	One grab sample of discharge on first day

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4.2 SAMPLE STORAGE AND PRESEVATION

Sample containers for large volumes of effluents should be either covered fiberglass or unsealed stainless steel tanks. Small volumes of effluent can be stored in reusable glass jugs or nonreusable Cubitainers or plastic "milk jugs."

Samples for on-site tests should be used immediately when practicable, but must be used within 36 hours of collection. It is generally not possible to refrigerate the large volume samples (200 liters or more) that are required for flow-through fish tests, but all other samples should be either iced or refrigerated if they are not to be used immediately.

Samples to be used for off-site tests should be iced for shipment and refrigerated (4•C) on receipt by the testing laboratory. As a minimum requirement in all cases, tests should be initiated within 36 hours of collection. In the case of short term chronic tests, samples taken on days one, three, and five may be held for a slightly longer period of time (up to 48 hours) to complete the test. In no case should any preservative be used in samples to be tested for toxicity.

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5. DILUTION WATER

The second major component discussed in detail is dilution water. The use of appropriate dilution water ensures that there will be a low number of responses in the controls and that any responses found in the solutions containing effluent are due to the effluent itself.

The choice of dilution water is generally specified in the NPDES permit and depends on the purpose of the toxicity test. Standard dilution water should be used to evaluate the inherent toxicity of the effluent. Dilution water from the receiving stream or a nontoxic equivalent should be used to test for interactions after discharge. Under most circumstances, however, the dilution water should not cause any toxic responses in test organisms. A lack of toxic responses in controls in the toxicity test is evidence of the suitability of the dilution water.

5.1 SOURCES OF DILUTION WATER

To determine the inherent toxicity of an effluent (e.g., for testing compliance with a toxicity limit), the source of water should be a standard dilution water made up from distilled water and known chemical compounds. The inspector should review the EPA manuals for details of obtaining dilution waters with various characteristics.

To determine the effects of an effluent on saltwater organisms, sea salts must be added to a freshwater effluent to attain the proper salinity. Dilution water could be hypersaline brine, an artificial mix (such as the commercial brands: 40 Fathoms® or Hawaiian Mix®), or natural seawater, depending on the specific recommendations for the protocol being used.

To determine the toxicity of an effluent relative to the receiving water, the dilution water should be taken from the receiving water as close as possible to, but outside the influence of, the

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outfall. If the test's objective is to determine the toxic contribution of the effluent to receiving waters, receiving waters are appropriate as dilution water -- whether they are contaminated or not. If the additive toxicity of the effluent to the receiving water is being established, receiving waters should be sampled daily as the source of dilution water.

With the estuarine (saline) receiving water, dilution waters of the same salinity as the receiving waters at the outfall should be used. If uncontaminated dilution water is required, waters from an adjacent estuary can be used or dilution water can be created with artificial sea salts. Alternatively, more saline waters (hypersaline brine) can be diluted to obtain dilution waters of the proper salinity.

If receiving waters are not available as dilution water, standard test waters, other surface waters, or ground water may be used. Waters other than receiving waters should be free from toxic effects (controls should have less than 10 percent mortality for acute tests and 20 percent mortality for chronic tests). Dechlorinated tap water should not generally be used as dilution water unless extensive precautionary steps are taken before its use. The EPA manuals describe techniques for treating tap water, but also discourage its use in favor of a standard dilution water or receiving waters.

5.2 STORAGE CONDITIONS AND HOLDING TIMES

Dilution water obtained from receiving waters should be immediately used for testing. If it is not used within 24 hours, it should be refrigerated (4°C) as soon as it is collected until it is used. In any case, it should be used within 36 hours of collection.

Notes:

6. TEST ORGANISMS

The third major component discussed in detail is the test organism. Since test organisms are the "analytical detection tool" to determine toxicity levels and since they can vary in their responses for reasons that are difficult to understand, special care must be devoted to their handling and treatment. Organisms used for toxicity testing are limited to certain species for which there are testing protocols. The life stage, source, acclimation and feeding procedures, presence of disease, and the number of organisms placed in test chambers all affect the degree to which organisms respond to toxicants. It is, thus, important that these factors are standardized as much as possible.

6.1 SPECIES USED

Tables 6-1 and 6-2 present the common name, scientific name, test temperature, and age range or life stage for those species for which there are acute toxicity testing protocols. Any of these species may be used in toxicity testing, but if a monitoring species is specified in a permit, that species must be used for all compliance monitoring for that effluent. If a toxicity limit is being determined for an effluent, EPA recommends that at least three species be used to determine which is most sensitive to the effluent.

Table 6-3 presents the common name, scientific name, test temperature, age range or life stage, and responses measured for all species for which there are short-term chronic toxicity testing protocols. Any of these species may be used in toxicity testing, but if a monitoring species is specified in a permit, that species must be used for all compliance monitoring for that effluent.

Notes:

TABLE 6-1.

FRESHWATER SPECIES FOR WHICH
THERE ARE ACUTE TOXICITY TESTING PROTOCOLS

FISH

COLD (12°C)			WARM (20°C)		
Common Name	Age/Life Stage	Scientific Name	Common Name	Age/Life Stage	Scientific Name
Brook trout	30-90 days	*Salvelinus fontinalis*	Bluegill	1-90 days	*Lepomis machrochirus*
Coho salmon	30-90 days	*Oncorhynchus kisutch*	Channel catfish	1-90 days	*Ictalurus punctatus*
Rainbow trout	30-90 days	*Salmo gairdneri*	Fathead minnow	1-90 days	**Pimephales promelas*

INVERTEBRATES

COLD (12°C)			WARM (20°C)		
Common Name	Age/Life Stage	Scientific Name	Common Name	Age/Life Stage	Scientific Name
Stone flies	Larvae	*Pteronarcys spp.*	Amphipods	Juveniles	*Hyalella spp.*
Crayfish	Juveniles	*Pacifastacus leniusculus*			*Gammarus lucustris*
Mayflies	Nymphs	*Baetis spp.,*			*G. fasciatus*
		Ephemerella spp.			*G. pseudolimnaeus*
			Cladocera	1-24 hr	*Daphnia magna*
					D. pluex
					**Ceriodaphnia dubia*
			Crayfish	Juveniles	*Orconectes spp.*
					Cambarus spp.
			Mayflies	Nymphs	*Hexagenia limbata*
					H. bilineata
			Midges	Larvae	*Chironomus spp.*

*Organisms may be tested at 25°C for both acute and short-term chronic tests.

Notes:

TABLE 6-2.

MARINE AND ESTUARINE SPECIES FOR WHICH THERE ARE ACUTE TOXICITY TESTING PROTOCOLS

FISH

COLD (12°C)			WARM (20°C)		
Common Name	Age/Life Stage	Scientific Name	Common Name	Age/Life Stage	Scientific Name
English sole	1-90 days	*Parophrys vetulus*	Flounder	1-90 days	*Paralichthys dentatus*
Sand dab	1-90 days	*Citharichthys stigmaeus*			*P. lethostigma*
			Longnose killfish	1-90 days	*Fundulus similis*
Winter flounder	Post-metamorphosis	*Pseudopleuronectes americanus*	Mummichog	1-90 days	*F. heteroclitus*
			Pinfish	1-90 days	*Lagodon rhomboides*
			Sheepshead minnow	1-90 days	*Cyprinodon variegatus*
			Silversides	1-90 days	**Menidia spp.*
			Spot	1-90 days	*Leiostomus xanthurus*
			Three-spined stickleback	1-90 days	*Gasterosteus aculeatus*

INVERTEBRATES

COLD (12°C)			WARM (20°C)		
Common Name	Age/Life Stage	Scientific Name	Common Name	Age/Life Stage	Scientific Name
Dungeness crab	Juvenile	*Cancer magister*	Blue crab	Juvenile	*Callinectes sapidus*
Oceanic shrimp	Juvenile	*Pandalus jordani*	Mysid	1-5 days	**Mysidopsis sp.*
Green sea urchin	Gametes/embryo	*Strongylocentrotus drobachiensis*			*Neomysis spp.*
			Grass shrimp	1-10 days	*Palaemonetes spp.*
Purple sea urchin	Gametes/embryo	*S. purpuratus*	Penеid shrimp	Post larval	*Peneus setiferus*
Sand dollar	Gametes/embryo	*Dendraster excentricus*			*P. duorarum*
			Sand shrimp	Post larval	*Crangon sp.*
			Pacific oyster	Post larval	*Crassostrea gigas*
			American oyster	Embryo/larval	*C. virginica*

*Organisms may be tested at 25°C for both acute and short term chronic tests.

Notes:

TABLE 6-3.

SPECIES FOR WHICH THERE ARE
CHRONIC TESTING PROTOCOLS, ORGANIZED BY SPECIES

Common Name	Response Measured	Temperature	Test Length	Age of Organism	Scientific Name
Fathead minnow	Larval survival and growth	25°C	7 days	Newly hatched larvae	*Pimephales promelas*
Fathead minnow	Embryo/larval survival and teratogenicity test	25°C	8 days	2-24 hour embryos	*Pimephales promelas*
Cladoceran	Survival and reproduction	25°C	7 days	>24 hour adults	*Ceriodaphnia spp.*
Algae	Growth	24°C	96 hours	4-7 day culture	*Selenastrum capricornutum*
Inland silverside	Larval survival and growth	25°C	7 days	7 days	*Menidia beryllina*
Sheepshead	Embryo survival and teratogenicity test	25°C	9 days	>24 hour larvae	*Cyprinodon variegatus*
Sheepshead	Larval survival and growth	25°C	7 days	>24 hour larvae	*Cyprinodon variegatus*
Mysids	Survival and growth	26°C	7 days	7 days	*Mysidopsis bahia*
Macroalgae	Reproduction	23°C	7 days	egg and sperm cells	*Champie parvula*
Sea Urchin	Fertilization	20°C	1-2 hours	egg and sperm cells	*Arbacia punctula*
Green Sea Urchin	Fertilization	12°C	1-2 hours	egg and sperm cells	*S. d roe bachiensis*
Purple Sea Urchin	Fertilization	12°C	1-2 hours	egg and sperm cells	*S. purpuratus*
Sand Dollar	Fertilization	12°C	1-2 hours	egg and sperm cells	*Dendraster excentrics*

Notes:

6.2 SOURCES OF TEST ORGANISMS

Although it might seem desirable to use organisms residing in the receiving water as test organisms, it is usually not practical for several reasons: (1) sensitive species might not be present; (2) it is usually difficult to find a sufficient number of organisms of the right age and condition; (3) special permits may be required in order to collect and use these species; (4) the exposure history to toxic chemicals is unknown; (5) they may be infested by parasites or diseases that can affect responses; and (6) they may have difficulty acclimating to test conditions.

Species most commonly used for test organisms are easily cultured. Laboratory cultures are, therefore, the best supply of organisms. However, the sensitivity of laboratory cultures must be tested periodically (once each month) with reference toxicants to ensure that the response of laboratory stocks is typical of other individuals of the same species.

If the organisms are not in culture, they may be obtained from commercial supply houses or government sources. However, each batch of organisms received from an outside source must be tested with reference toxicants to ensure that their responses are typical of other individuals of the same species.

If it is decided to collect organisms from the wild, they must be observed over a period of time to ensure that they do not become diseased or lose condition due to being held in the laboratory. Wild organisms should be tested with reference toxicants to ensure their responses are typical of other individuals of the same species, where known.

6.3 ACCLIMATION AND FEEDING

Test organisms are normally kept in culture in water and at temperatures that may be different from the condition and temperature of the dilution water for a test. Culture waters are chosen because they are readily available at a laboratory at low cost; temperatures are chosen to

Notes:

ensure maximum perpetuation of stocks in the chosen water. Since changes in temperature and changes in key water parameters can have an apparent toxic effect on test organisms, organisms must be acclimated to test conditions prior to testing.

6.3.1 Acclimation

Acclimation is achieved by slowly adding dilution water over 24 to 48 hours to the water in which organisms are cultured or held. When organisms are in waters substantially different from the dilution water, the rate of change of conditions between the storage water and dilution water should be limited. Organisms commonly are stressed by changes in temperature of greater than 3°C or changes of salinity of greater than 3 ppt in any 12-hour period so changes during acclimation should not exceed these rates. In addition, changes in pH or alkalinity should be made slowly to allow organisms to adapt. If mortality of the organisms exceeds 5 percent in the 24 hours immediately preceding initiation of the test (even if this includes the acclimation period), a new batch of test organisms should be obtained. If a new batch of organisms has 10 percent mortality in the 24 hours preceding a test in the same dilution water, a different dilution water should be used. Acclimation for each of the EPA chronic tests is discussed in the chronic manuals.

6.3.2 Feeding

Feeding test organisms during testing is sometimes necessary, depending on the species used and the test's length. Recommended feeding procedures for each test and species are outlined in the EPA manuals previously cited in Chapter 1. With static tests, excess food must be removed as soon as possible after feeding to ensure that bacterial decomposition does not foul the water or reduce oxygen concentrations in the test solutions, except in the case of Ceriodapnia, which must be fed after the adult is transferred to the fresh test solution. With static renewal tests, feeding should occur just prior to renewal of the test solutions. Feeding during flow-through tests does not usually pose significant problems, but adding much more food than is necessary to maintain the health of test organisms is not recommended. Whenever possible, adequate amounts of nutritional

Notes:

food must be provided to maintain normal growth rates of test organisms during testing.

6.4 DISEASE

An obviously diseased or discolored organism should not be used in toxicity tests since either of these conditions may cause the organism to be more sensitive to toxicant stress. If disease develops during the course of a toxicity test (particularly if it develops in the controls as well as lower concentrations of the effluent), the test should be terminated and the results discarded.

6.5 LOADING RATES

Organisms in test chambers consume oxygen and excrete potentially toxic materials during a toxicity test. It is important to control the number of organisms so that effects caused by their presence can be minimized. For this reason, it is recommended that the weight of organisms in test chambers should not exceed 5 g/l of test solution for flow-through tests at 20°C or colder, and 2.5 g/l for flow-through tests above 20°C. In static and static renewal tests, organism weight should not exceed 0.8 g/l of test solution at 20°C or less, and 0.4 g/l above 20°C. Loading rates for each of the chronic tests are listed in the chronic manuals.

Further, the recommended loading rate should not cause the dissolved oxygen concentration in the test chambers to be reduced below 40 percent saturation[1] for test temperatures above 20°C or 60 percent saturation for test temperatures at 20°C or less. However, if oxygen concentrations approach these limits due to bacterial action in the test solutions, aeration may be necessary to prevent low oxygen levels from adversely affecting the test organisms. Aeration should be minimized to prevent loss of volatile toxics.

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[1] For saltwater tests with *Menidia* sp., oxygen concentrations may need to exceed 40 percent saturation.

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7. TEST SYSTEM

The fourth component discussed in detail is the test system. This component is the most complicated to explain because it is closely tied to the procedures used in toxicity testing. The test system includes a number of specific items, such as:

- Equipment used for storing effluents and dilution water prior to use
- Chambers where effluent and dilution water are mixed in the appropriate concentrations
- Test chambers where organisms are exposed to the effluent/dilution water mixtures
- Tubing, valves, or pumps through which effluent or dilution water passes
- Test conditions.

The first four items listed above are discussed together since the important considerations for these items are the materials of which they are constructed and their cleanliness during a toxicity test. Test conditions are dealt with separately at the end of this section.

7.1 MATERIALS USED

Any material that comes into contact with either effluent or dilution water must not release, absorb, or adsorb toxicants. A number of different choices for this material is available. Glass and No. 304 or 306 stainless steel are generally acceptable for freshwater holding, mixing, and test chambers. Stainless steel, however, is not acceptable for saltwater systems. Square-sided glass aquaria should be held together with small beads of silicone adhesive, with any unnecessary adhesive removed from inside the aquaria. If stainless steel containers are used, they must be welded, not

Notes:

soldered. Other specialized containers of NITEX or TEFLON are also acceptable. Tanks for holding effluents and dilution water may also be made of fiberglass. All containers or tubes made of these materials are reusable with appropriate cleaning (see next section).

Polyethylene, polypropylene, polyvinyl chloride, polystyrene, and TYGON may also be used for containers or tubing, but should be checked for toxicity before being used. Because these materials may absorb toxicants during a test, their reuse is discouraged to prevent absorbed toxicants from leaching into new effluent or dilution water.

Copper, galvanized metal, brass, lead, and rubber must not contact the testing solutions at any time.

7.2 CLEANING

New plasticware (from a known nontoxic source) can be used after rinsing with dilution water. New glassware should be soaked overnight in dilute (20 percent V:V) nitric or hydrochloric acid, rinsed in tap water, and then rinsed with dilution water before use.

Glassware and stainless steel components should be soaked in detergent and scrubbed (or washed in a laboratory dishwater), rinsed twice with tap water, rinsed with dilute acid, rinsed twice with tap water, rinsed with full-strength acetone, rinsed twice with tap water, and then rinsed with dilution water before use. Glassware for algae tests should also be neutralized in sodium bicarbonate before use.

7.3 TEST CONDITIONS

There are several physical/chemical measurements which are done in conjunction with whole effluent toxicity tests to ensure the conditions of the test are within acceptable ranges for

Notes:

maintenance of the test organisms. Three key parameters must be measured during a test to ensure comparability of results between tests:

- Temperature
- Oxygen levels
- Salinity (for marine tests only).

Other parameters are measured because of their relationships to effluents and toxicants:

- pH
- Total alkalinity (fresh-water tests only)
- Total hardness (fresh-water tests only)
- Conductivity (fresh-water tests only)
- Total residual chlorine (fresh-water tests only).

Each of these parameters is discussed in the following subsections.

7.3.1 Temperature

Each toxicity test has a nominal temperature and a range of temperatures (usually ± 1° or 2°C) over which the test should be run. Since organisms may be more sensitive to toxicants at temperatures approaching their tolerance limits, these temperature ranges are critical in standardizing responses. The results of tests with ambient temperatures outside of the acceptable range (in either direction) should be carefully reviewed for acceptability.

Notes:

Temperatures for static tests can be controlled by placing the test apparatus in a controlled environment room or incubator at the appropriate temperature. Temperature control is also achieved using circulating water baths. The latter provides more constant temperatures and is less likely to be affected by external factors such as lights and variations in air temperatures. Temperatures of environment rooms or water baths should be calibrated with reference thermometers periodically (when changes are suspected or at least once per month).

7.3.2 Oxygen Levels

Because low dissolved oxygen (DO) levels produce stress in test organisms, DO levels should not decline below 4 mg/l DO for tests above 20°C or 6 mg/l DO for tests below 20°C. Generally, it is best to run flow-through tests if low DO is expected to occur because flow-through tests allow continuous replenishment of the test solutions before DO can decline in the test chambers. However, at higher concentrations of effluent, oxygen may decline even in flow-through systems, so aeration may be required.

Aeration may affect the toxicity of effluents and should be used only as a last resort. Aeration, if applied, should be uniform across all test vessels. If aeration is required in a flow-through system, it should initially be provided in the dilution water, and if low DO still occurs in test chambers, each test chamber should also be aerated. Aeration for individual test chambers should be provided through a pipette, not an air stone. Air flow through the bubbler should be at the lowest rate necessary to maintain DO levels, but should not exceed 100 bubbles per minute. Agitation caused by more rapid bubble rates can drive off volatile toxicants in the test system.

7.3.3 Salinity

Salt-water tests require strict adherence to allowable salinity ranges. Test dilutions which fall outside of the specified range may give inaccurate test results. Likewise, salinity should vary by no more than ± 2 parts per thousand among the chambers (all dilutions) on a given day.

Notes:

Salinity levels may be achieved by the use of dilution water which is natural sea water, hypersaline brine prepared from natural sea brine, or (if recommended in the specific protocol) artificial seawater prepared from sea salts. The selection of dilution water may limit the maximum concentration of effluent that can be used in the test.

7.3.4 pH

Because of the effect pH has on the test organisms and the effect that it has on the toxicity of some compounds (e.g., ammonia toxicity increases as the pH rises above 7), pH should be monitored daily. If necessary, tests can be run in corked vessels in which gaseous CO_2 has been introduced above the test solution to control pH rise.

7.3.5 Total Alkalinity, Total Hardness, Conductivity, and Total Residual Chlorine

Several chemical measurements are recommended for the first day of the test to aid in the interpretation of results, or at least to provide leads into further investigations if toxicity exists. Total alkalinity will indicate the buffering capacity of the waste; hardness and conductivity can indicate the magnitude of dissolved solids in the waste, and potentially indicate variability in effluent quality. Total Residual Chlorine is a recommended analysis because of the prevalence of this toxicant in POTW wastewaters and its lethality in low concentrations.

7.4 TEST REPLICATES

In order to determine statistically the significance of the results for any concentration of effluent, it is necessary to test a sufficient number of organisms with each concentration and the control. The protocol manuals specify the minimum number of replicates and organisms required for each method. This information is also summarized in the summary tables in Appendix B.

Notes:

8. TEST RESULTS

The final component of toxicity testing discussed in detail is the test result. The valid interpretation of test results requires that mortality in controls is limited, the conditions specified in the previous sections are met, and the results are consistent with response patterns normally observed in toxicity tests (e.g., increasing mortality with increasing concentrations). If any of these three general conditions are not met, the inspector should not calculate summary statistics and should ignore test results.

8.1 CONTROL SURVIVAL

In general, survival in controls must exceed survival in all other test chambers for both acute and chronic tests. If it does not, calculation of the toxicity due to increasing effluent concentration is at best an approximation of effluent toxicity. In any case, mortality in controls should not exceed 10 percent for acute toxicity tests and 20 percent for chronic tests (or other values as required by States through their regulations). If control survival does not meet 90 or 80 percent for an acute or chronic test, respectively, then results should not be used for calculating summary statistics, and a determination of compliance using the test results cannot be made.

8.2 ACCEPTABILITY CRITERIA

Each protocol has specified criteria for acceptable ranges of control survival, temperature, dissolved oxygen concentration, salinity, pH, light intensity and duration of photoperiod, organism loading (numbers or weight per volume), feeding, and cleaning procedures. Summary tables of each of the EPA methods is in Appendix B of this module. Tests not meeting the control criteria for survival, growth, or reproduction are not valid. Tests not meeting the other acceptability criteria in these tables should be reviewed with caution and referred to the Regional Biologist. The inspector should review the EPA methods manual for a more extensive discussion of each of these factors.

Notes:

8.3 RESULTS CALCULATION

The expected result in all toxicity tests is a greater number of organism responses with increasing effluent concentrations. On many occasions, this increasing response is observed as one concentration eliciting no responses and the next higher concentration having 100 percent responses. This pattern is particularly obvious with acute tests. In other cases, the test organisms in more than one effluent dilution may exhibit a partial response (between 0 and 100 percent).

When test results do not meet the expected pattern, the test may be invalid. Questionable results in an acute test include:

- Higher mortalities in lower concentrations than in higher concentrations of effluent
- 100 percent mortality in all effluent dilutions
- Greater percent mortality in the control than in the lower dilutions of effluent.

Questionable results in a chronic test include:

- Greater growth or reproduction or fewer terata at higher concentrations of effluent than at lower concentrations
- No growth or reproduction or 100 percent terata at all effluent concentrations
- Less growth or reproduction or more terata in controls than in lower effluent concentrations.

When any of these results occur (outside of experimental error), the results and test conditions should be reviewed by the Regional biologist. It should be recognized, however, that

Notes:

often there will be minor variations in test results. For example, *Ceriodaphnia dubia* reproduction may be higher at intermediate concentrations which are not toxic but provide a greater food resource than lower concentrations. Thus, variations should not always be used to eliminate otherwise valid results. However, if the normally expected pattern is not found, summary statistics calculated on the results should be assessed with caution.

Under some circumstances, compliance may still be determined with abnormal test results. If, for example, 100 percent responses were found in all effluent dilutions but the control was within the acceptable response range, the appropriate toxicity measure must be below the most dilute solution tested. Similarly, if no responses are found in the toxicity test, the effluent can be deemed nontoxic at 100 percent effluent.

Methods and computer programs by which summary statistics can be calculated are listed in the manuals. Each of the methods or programs can be used only under limited circumstances. If these circumstances are not met, the results calculated will be erroneous. Make sure that the assumptions specified for each analysis are appropriate for the data being analyzed before using these programs.

Notes:

APPENDIX A

QUESTIONS AND ANSWERS ON THE BIOMONITORING MODULE

QUESTIONS ON THE BIOMONITORING MODULE

1. A toxicity test in which the effluent and dilution water are continuously replenished in test chambers is called a _____ test.

2. A _____ toxicity test estimates the concentration at which a predetermined toxic response occurs.

3. The _____ is the concentration at which 50 percent of the test organisms die in a specified length of time.

4. The highest concentration at which test organisms show no responses in a chronic test is called the _____.

5. Toxicity is _____.

6. The ChV, _____, is calculated by _____.

7. Effluent samples should be taken _____. (where)

8. If an effluent sample is not to be used immediately, how should it be preserved?
   _____

9. Small volume effluent samples should be stored in _____, _____, or _____ containers.

10. Tanks for storing large volume effluent samples should be made of _____ or _____.

11. Effluent samples should be used within _____ hours for on-site testing and _____ hours for off-site testing.

12. Effluent samples shipped to a laboratory for off-site testing should be preserved by being shipped _____ and _____ on receipt by the lab.

13. Nonpersistant effluent samples must be used within _____ hours of sampling.

14. Effluent samples for flow-through and static renewal tests are taken at least _____ for the duration of the test.

15. Continuous discharges with retention times of less than 14 days must be sampled _____ for the duration of the test.

16. What should be used as dilution water for a test to determine the inherent toxicity of an effluent? _____

17. What should be used as dilution water for a test to determine the relative toxicity of an effluent in relation to receiving waters? _____

18. Mortality in controls must be less than _____ percent for acute tests and less than _____ for chronic tests.

19. Test organisms are suitable for use in a toxicity test when there is less than _____ percent mortality during acclimation.

20. Receiving water that is used as dilution water should be refrigerated if not used in a test within _____ hours.

21. A new sample of receiving water should be taken if the sample is not used as dilution water within _____ hours of sampling.

22. The two species most commonly used in toxicity testing are _____ and _____.

23. The species to be used in testing for compliance monitoring is _____.

24. How frequently should the response of organisms receiving from supply houses be tested with reference toxicants? _____

25. How frequently should the response of organisms raised in laboratory culture be tested with reference toxicants? _____

26. Organisms whose responses in a test with a reference toxicant are outside the limits specified for that toxicant _____.

27. Adding dilution water to the water that organisms were raised in prior to a test is called _____.

28. Test organisms should be fed types of food and in amounts that are_____ for each species and test protocol.

29. Test organisms showing signs of disease should _____.

30. Only (weight) of test organisms should be added per liter of test solution for warm water static tests.

31. Test chambers may be made of _____, _____, and _____.

32. Plastic containers and tubing that are used in a toxicity test _____.

33. No materials containing _____, _____, _____, _____, and _____ should come in contact with any solution to be used in toxicity testing.

34. New glassware should be _____ before use in toxicity testing.

35. Before use in another toxicity test, stainless steel containers and glassware should be _____.

36. Test results obtained when temperatures were outside the ranges specified by the test protocol _____.

37. DO should be above _____ percent saturation for tests run at more than 20°C or above _____ percent saturation for tests run at 20°C or lower.

38. Aeration should be provided only by _____.

39. Typical toxicity tests show a higher number of responses with _____ concentrations of effluent.

ANSWERS ON THE BIOMONITORING MODULE

1. Flow-through

2. Definitive

3. LC_{50}

4. NOEC (no observable effect concentration)

5. A characteristic of a substance that causes adverse responses in organisms

6. The chronic value, taking the geometric mean of the NOEC and LOEC

7. At the point specified in a permit

8. It should be refrigerated at 4°C or placed on ice

9. Cubitainers, plastic milk jugs, or glass containers

10. Fiberglass or stainless steel

11. 24 hours on-site, 76 hours off-site

12. On ice, refrigerated

13. 36

14. Daily

15. Twice daily

16. Standard dilution water (artificial water)

17. Receiving water

18. 10 for acute, 20 for chronic

19. 5

20. 24

21. 96

22. *Ceriodaphnia dubia* and *Pimephales promelas* or the water flea and the fathead minnow

23. The species identified in the permit

24. On receipt of each batch

25. At least once each month

26. Should not be used in toxicity testing

27. Acclimation

28. Recommended in the manuals

29. Not be used in toxicity testing

30. 0.4 g

31. Plastic that has been tested for toxicity, glass, and stainless steel

32. Should be discarded

33. Copper, brass, galvanized metal, rubber, or lead

34. Soaked in acid and rinsed thoroughly

35. Rinsed with acid, rinsed with tap water, rinsed with acetone, rinsed with tap water then rinsed with dilution water

36. Should be carefully evaluated for acceptability

37. 40, 60

38. Bubbling air through a pipette at less than 100 bubbles per minute

39. Higher.

APPENDIX B

DATA SHEETS FOR AQUATIC TOXICITY TESTS:

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR SOME COMMONLY USED TEST SPECIES

TABLE 1.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR DAPHNID
(Daphnia pulex AND Daphnia magna) ACUTE TOXICITY TEST

1.	Temperature:	20 ± 2°C
2.	Light quality:	Ambient laboratory illumination
3.	Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient lab levels)
4.	Photoperiod:	8-16 hours light/24 hours
5.	Test chamber size:	100 ml beaker or equivalent
6.	Test solution volume:	50ml/replicate (loading and DO must be met)
7.	Age of test organisms:	1-24 hour (neonates)
8.	Neonates/test chamber:	10
9.	Replicate chambers/concentration:	2
10.	Total number of organisms per concentration:	20
11.	Feeding regime:	Feeding not required during first 48 hour. For longer tests, feed every other day beginning on the third day.
12.	Aeration:	None, unless DO falls below 40% of saturation, at which time start gentle, single-bubble, aeration.
13.	Dilution water:	Receiving water or other surface water, ground water, or synthetic water: hard water for *Daphnia magna*; moderately hard or soft water for *Daphnia pulex*
14.	Test duration:	Screening test - 24 h (static test) Definitive test - 48 h (static test)
15.	Effects measured:	Mortality - no movement of body or appendages on gentle prodding (LC_{50})
16.	Test acceptability:	Control survival of 90% or greater

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

TABLE 2.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR MYSID (Mysidopsis bahia) ACUTE TOXICITY TEST

1.	Temperature:	20 ± 2°C
2.	Light quality:	Ambient laboratory illumination
3.	Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient lab levels)
4.	Photoperiod:	8-16 hours light/24 hours
5.	Test chamber size:	250 ml beaker or equivalent
6.	Test solution volume:	200ml/replicate (loading and DO restrictions must be met)
7.	Age of test organisms:	1-5 days
8.	Organisms/test chamber:	10
9.	Replicate chambers/concentration:	2
10.	Total number of organisms per concentration:	20
11.	Feeding regime:	Two drops of concentrated brine shrimp nauplii suspension twice daily (approx. 100 nauplii/mysid)
12.	Aeration:	None, unless DO falls below 40% of saturation, at which time start gentle, single-bubble, aeration.
13.	Dilution water:	Natural seawater, or synthetic salt water adjusted to 20 ppt salinity
14.	Test duration:	Screening test - 24 h (static test) Definitive test - 48 h (static test); 48-96 h (flow-thru)

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

15. Effects measured:	Mortality - no movement of body or appendages on gentle prodding (LC_{50})
16. Test acceptability:	Control survival of 90% or greater.

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

TABLE 3.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR FATHEAD MINNOW (Pimephales promelas) ACUTE TOXICITY TEST

1.	Temperature:	20 ± 2°C
2.	Light quality:	Ambient laboratory illumination
3.	Light intensity:	10-20 μE/m²/s (50-100 ft-c) (ambient lab levels)
4.	Photoperiod:	8-16 hours light/24 hours
5.	Test chamber size:	1 L beaker or equivalent
6.	Test solution volume:	0.75 L/replicate (loading and DO restrictions must be met)
7.	Age of test organisms:	1-90 days
8.	Number of fish/test chamber:	10
9.	Replicate chambers/concentration:	2
10.	Total number of organisms per concentration:	20
11.	Feeding regime:	Feeding not required first 96 h
12.	Aeration:	None, unless DO falls below 40% of saturation, at which time start gentle, single-bubble, aeration.
13.	Dilution water:	Receiving water, other surface water, ground water, or soft synthetic water
14.	Test duration:	Screening test - 24 h (static test) Definitive test - 48 h (static test); 48-96 h (flow-thru)

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

15. Effects measured:	Mortality - no movement (LC_{50})
16. Test acceptability:	Control survival of 90% or greater.

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

TABLE 4.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR SILVERSIDE (Menidia spp.) ACUTE TOXICITY TEST

1.	Temperature:	20 ± 2°C (northern latitudes) 25 ± 2°C (southern latitudes)
2.	Light quality:	Ambient laboratory illumination
3.	Light intensity:	10-20 μE/m²/s (50-100 ft-c) (ambient lab levels)
4.	Photoperiod:	8-16 hours light/24 hours
5.	Test chamber size:	1 L beaker or equivalent
6.	Test solution volume:	0.75 L/replicate (loading and DO restrictions must be met)
7.	Age of test organisms:	1-90 days
8.	Organisms/test chamber:	10
9.	Replicate chambers/concentration:	2
10.	Total number of organisms per concentration:	20
11.	Feeding regime:	Feeding not required first 96 h
12.	Aeration:	None, unless DO falls below 40% of saturation, at which time start gentle, single-bubble, aeration.
13.	Dilution water:	Natural seawater, or synthetic salt water adjusted to 25-30 ppt salinity
14.	Test duration:	Screening test - 24 h (static test) Definitive test - 48 h (static test); 48-96 h (flow-thru)

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

15. Effects measured:	Mortality - no movement (LC_{50})
16. Test acceptability:	Control survival of 90% or greater.

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013. March 1985.

TABLE 5.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR FATHEAD MINNOW (Pimephales promelas) LARVAL SURVIVAL AND GROWTH TEST

1.	Test type:	Static renewal
2.	Temperature:	25°C ± 1°C
3.	Light quality:	Ambient laboratory illumination
4.	Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient lab levels)
5.	Photoperiod:	16 hours light, 8 hours darkness
6.	Test chamber size:	500 ml beakers or equivalent
7.	Test solution volume:	250 ml/replicate (loading and DO restrictions must be met)
8.	Renewal of test concentrations:	Daily
9.	Age of test organisms:	Newly hatched larvae (less than 24 hours old)
10.	Larvae/test chamber:	15 larvae/chamber (minimum 10)
11.	Replicate chambers/concentration:	4 (minimum of 3)
12.	Larvae/concentration:	60 larvae/concentration (minimum 30)
13.	Feeding regime:	Feed 0.1 ml newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals or, as a minimum, 0.15 ml twice daily, 6 h between feedings (at the beginning of the work day prior to renewal, and at the end of the work day following renewal). Sufficient larvae are added to provide an excess. Larvae are not fed during the final 12 h of the test.
14.	Cleaning:	Siphon daily, immediately before test solution renewal
15.	Aeration:	None, unless DO falls below 40% of saturation. Rate should not exceed 100 bubbles/min.

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

16.	Dilution water:	Moderately hard synthetic water is prepared using Millipore Milli-Q[R] or equivalent deionized water and reagent grade chemicals or 20% DMW
17.	Effluent concentrations:	Minimum of 5 and a control
18.	Dilution factor:	Approximately 0.3 or 0.5
19.	Test duration:	7 days
20.	Endpoints:	Survival and growth (weight)
21.	Test acceptability:	80% or greater survival in controls; average dry weight of surviving controls equals or exceeds 0.25 mg
22.	Sampling requirement:	For on-site tests, samples are collected daily, and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples are collected, and used on days 1-2, 3-4, and 5-7.
23.	Sample volume required:	2.5 L/day

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

TABLE 6.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR FATHEAD MINNOW (Pimephales promelas) EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST

1.	Test type:	Static renewal
2.	Temperature:	25°C ± 1°C
3.	Light quality:	Ambient laboratory illumination
4.	Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient lab levels)
5.	Photoperiod:	16 hours light, 8 hours darkness
6.	Test chamber size:	150-500 ml beakers or equivalent
7.	Test solution volume:	70-200 ml/replicate (loading and DO restrictions must be met)
8.	Renewal of test concentrations:	Daily
9.	Age of test organisms:	Less than 36 hour old embryos
10.	Larvae/test chamber:	15 larvae/chamber (minimum 10)
11.	Replicate chambers/ concentration:	4 (minimum of 3)
12.	Larvae/concentration:	60 larvae/concentration (minimum 30)
13.	Feeding regime:	Feeding not required
14.	Aeration:	None, unless DO falls below 40% of saturation. Rate should not exceed 100 bubbles/min.
15.	Dilution water:	Moderately hard synthetic water is prepared using Millipore Milli-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW. The hardness of the test solutions must equal or exceed 25 mg/L ($CaCO_3$) to ensure hatching.

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

16. Effluent concentrations:	Minimum of 5 and a control
17. Dilution factor:	Approximately 0.3 or 0.5
18. Test duration:	7 days
19. Endpoint:	Combined mortality (dead and deformed organisms)
20. Test acceptability:	80% or greater survival in controls
21. Sampling requirement:	For on-site tests, samples are collected daily, and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples are collected, and used on days 1-2, 3-4, and 5-7.
22. Sample volume required:	2.5 L/day

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

TABLE 7.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR
Ceriodaphnia SURVIVAL AND REPRODUCTION TEST

1.	Test type:	Static renewal
2.	Temperature:	25•C ± 1•C
3.	Light quality:	Ambient laboratory illumination
4.	Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient lab levels)
5.	Photoperiod:	16 hours light, 8 hours darkness
6.	Test chamber size:	30 ml beakers or equivalent
7.	Test solution volume:	15 ml/replicate (loading and DO restrictions must be met)
8.	Renewal of test concentrations:	Daily
9.	Age of test organisms:	Less than 24 hour; all released within a 8-h period
10.	Neonates/test chamber:	1
11.	Replicate chambers/concentration:	10
12.	Neonates/concentration:	10
13.	Feeding regime:	Feed 0.1 ml each of YCT and algal suspension per test chamber daily
14.	Aeration:	None
15.	Dilution water:	Moderately hard synthetic water is prepared using Millipore Milli-Q^R or equivalent deionized water and reagent grade chemicals or 20% DMW.
16.	Effluent concentrations:	Minimum of 5 and a control
17.	Dilution factor:	Approximately 0.3 or 0.5

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

18. Test duration:	Until 60% of control females have three broods (may require more or less than 7 days).
19. Endpoint:	Survival and reproduction
20. Test acceptability:	80% or greater survival and an average of 15 or more young/surviving female in controls. At least 60% of surviving females in controls should have produced their third brood.
21. Sampling requirement:	For on-site tests, samples are collected daily, and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples are collected, and used on days 1-2, 3-4, and 5-7.
22. Sample volume required:	1 L/day

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

TABLE 8.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR THE ALGAL (Selenastrum capricornutum) GROWTH TEST

1.	Test type:	Static
2.	Temperature:	25•C ± 1•C
3.	Light quality:	"Cool white" fluorescent lighting
4.	Light intensity:	86 ± 8.6 $\mu E/m^2/s$ (400 ± 40 ft-c)
5.	Photoperiod:	Continuous illumination
6.	Test chamber size:	125 or 250 ml beakers or equivalent
7.	Test solution volume:	50 or 100 ml/replicate
8.	Renewal of test concentrations:	None
9.	Age of test organisms:	4 to 7 days
10.	Initial cell density:	10,000 cells/ml
11.	Replicate	ðhambers/conc.:
12.	Shaking rate:	100 cpm continuous, or twice daily by hand
13.	Dilution water:	Algal stock culture medium without EDTA or enriched surface water
14.	Effluent concentrations:	Minimum of 5 and a control
15.	Dilution factor:	Approximately 0.3 or 0.5
16.	Test duration:	96 h
17.	Endpoint:	Growth (cell counts, chlorophyll fluorescence, absorbance, biomass)

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

18. Test acceptability:	2 X 10^5 cells/ml in the controls; variability of controls should not exceed 20%
19. Sample volume required:	1 L (one sample for test initiation)

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

TABLE 9.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR SHEEPHEAD MINNOW (Cyprinodon variegatus) LARVAL SURVIVAL AND GROWTH TEST

1.	Test type:	Static renewal
2.	Salinity:	20ppt to 32ppt ± 2ppt
3.	Temperature:	25°C ± 2°
4.	Light quality:	Ambient laboratory illumination
5.	Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient lab levels)
6.	Photoperiod:	14 hours light, 10 hours darkness
7.	Test chamber size:	300ml - 1L beakers or equivalent
8.	Test solution volume:	250-750ml/replicate (loading and DO restrictions must be met)
9.	Renewal of test concentrations:	Daily
10.	Age of test organisms:	Newly hatched larvae (less than 24 hours old)
11.	Larvae/test chamber:	15 larvae/chamber (minimum 10)
12.	Replicate chambers/concentration:	4 (minimum of 3)
13.	Source of food:	Newly hatched *Artemia nauplii* (less than 24 hours old)
14.	Feeding regime:	Feed once a day 0.10g wet weight *Artemia nauplii* per replicate on Days 0-2; feed 0.15g wet weight *Artemia nauplii* per replicate on Days 3-6
15.	Cleaning:	Siphon daily, immediately before test solution renewal
16.	Aeration:	None, unless DO falls below 60% of saturation, then aerate all chambers. Rate should be less than 100 bubbles/minute

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

17.	Dilution water:	Uncontaminated source of natural seawater, hypersaline brine, or artificial seawater mixed with deionized water
18.	Effluent concentrations:	5 and a control
19.	Dilution factor:	Approximately 0.3 or 0.5
20.	Test duration:	7 days
21.	Effects measured:	Survival and growth (weight)
22.	Test acceptability:	Control survival of 80% or greater, and average control average dry weight of 0.6 mg or greater or, if preserved, 0.5 mg or greater.

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

TABLE 10.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR SHEEPHEAD MINNOW (Cyprinodon variegatus) EMBRYO LARVAL SURVIVAL AND TERATOGENICITY TEST

1.	Test type:	Static renewal
2.	Salinity:	5ppt to 32ppt ± 2ppt
3.	Temperature:	25°C ± 2°
4.	Light quality:	Ambient laboratory illumination
5.	Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient lab levels)
6.	Photoperiod:	14 hours light, 10 hours darkness
7.	Test chamber size:	500 ml
8.	Test solution volume:	400 ml (minimum of 250 ml)
9.	Renewal of test concentrations:	Daily
10.	Age of test organisms:	less than 24 hours old
11.	Embryos/test chamber:	15 embryos/chamber (minimum 10)
12.	Replicate chambers/ concentration:	4 (minimum of 3)
13.	Embryos per concentration:	60 (minimum of 30)
14.	Feeding regime:	Feeding not required
15.	Aeration:	None, unless DO falls below 60% of saturation
16.	Dilution water:	Uncontaminated source of natural seawater, hypersaline brine, or artificial seawater mixed with deionized water
17.	Effluent concentrations:	5 and a control
18.	Dilution factor:	Approximately 0.3 or 0.5

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

19. Test duration:	9 days
20. Effects measured:	Percent hatch; percent larvae dead or with debilitating morphological and/or behavior abnormalities such as: gross deformities, curving spine, disoriented, abnormal swimming behavior; surviving normal larvae from original embryos
21. Test acceptability:	Control survival of 80% or greater.

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

TABLE 11.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR THE INLAND SILVERSIDE (Menidia beryllina) LARVEL SURVIVAL AND GROWTH TEST

1.	Test type:	Static renewal
2.	Salinity:	5ppt to 32ppt ± 2ppt
3.	Temperature:	25•C ± 2•
4.	Light quality:	Ambient laboratory illumination
5.	Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient lab levels)
6.	Photoperiod:	14 hours light, 10 hours darkness
7.	Test chamber size:	300ml - 1L containers
8.	Test solution volume:	250-750ml/replicate (loading and DO restrictions must be net)
9.	Renewal of test concentrations:	Daily
10.	Age of test organisms:	7 - 11 days post hatch
11.	Larvae/test chamber:	15 larvae/chamber (minimum 10)
12.	Replicate chambers/ concentration:	4 (minimum of 3)
13.	Source of food:	Newly hatched *Artemia nauplii*
14.	Feeding regime:	Feed 0.10g wet weight *Artemia nauplii* per replicate on days 0-2; Feed 0.15g wet weight *Artemia nauplii* per replicate on days 3-6
15.	Cleaning:	Siphon daily, immediately before test solution renewal and feeding
16.	Aeration:	None, unless DO falls below 60% of saturation, then aerate all chambers. Rate should be less than 100 bubbles/minute

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

17. Dilution water:	Uncontaminated source of natural seawater or hypersaline brine mixed with deionized water
18. Effluent concentrations:	At least 5 and a control
19. Dilution factor:	Approximately 0.3 or 0.5
20. Test duration:	7 days
21. Effects measured:	Survival and growth (weight)
22. Test acceptability:	Control survival of 80% or greater, control average dry weight (for 7 day old larvae) of 0.5 mg or greater, or, if preserved, 0.43 mg or greater.

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

TABLE 12.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR THE Mysidopsis bahia 7-DAY SURVIVAL GROWTH, AND FECUNDITY TEST

1.	Test type:	Static renewal
2.	Salinity:	20ppt to 30ppt ± 2ppt
3.	Temperature:	26°-27°C
4.	Light intensity:	10-20 μE/m²/s (50-100 ft.c.) (ambient lab levels)
5.	Photoperiod:	16 h light, 8 h darkness, with phase in/out period
6.	Test chamber:	8oz plastic disposable cups or 400ml glass beakers
7.	Test solution volume:	150ml/replicate cup
8.	Renewal of test solutions:	Daily
9.	Age of test organisms:	7 days
10.	Organisms/test chamber:	5
11.	Replicate chambers/ treatment:	8
12.	Source of food:	*Artemia nauplii*
13.	Feeding regime:	Feed 150 24-h old *Artemia nauplii* per mysid daily, half after test solution renewal and half after 8-12 hours
14.	Cleaning:	Pipette excess food from cups daily
15.	Aeration:	None, unless DO falls below 60% of saturation, then gently in all cups
16.	Dilution water:	Uncontaminated source of natural seawater or hypersaline brine
17.	Number of treatments/study:	Minimum of 5 and a control

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

18. Dilution factor:	Approximately 0.3 or 0.5
19. Test duration:	7 days
20. Effects measured:	Survival, growth, and egg development
21. Test acceptability:	Control survival of 80% or greater, control dry weight of 0.2 mg/mysid or greater.

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

TABLE 13.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR
Arbacia punctulata FERTILIZATION TEST

1.	Test type:	Static
2.	Salinity:	30ppt ±2ppt
3.	Temperature:	20°±1°C
4.	Light quality:	Ambient laboratory light during test preparation
5.	Light intensity:	10-20 μE/m²/s (50-100 ft.c.) (ambient lab levels)
6.	Test vessel size:	Disposable (glass) liquid scintillation vials (20ml capacity), not pre-cleaned
7.	Test solution volume:	5ml
8.	Number of sea urchins:	Pooled sperm from four males and pooled eggs from four females per test
9.	Number of egg and sperm cells per chamber:	About 2000 eggs and 5,000,000 sperm cells per vial
10.	Replicate chambers/ treatment:	4 (minimum of 3)
11.	Dilution water:	Uncontaminated source of natural seawater or deionized water mixed with hypersaline brine or artificial sea salts
12.	Dilution factor:	0.3 or 0.5
13.	Number of treatments/study:	Minimum of 5 effluent concentrations and a control
14.	Test duration:	1 hour and 20 minutes
15.	Effects measured:	Fertilization of sea urchin eggs
16.	Test acceptability:	Control fertilization sperm:egg ratio of 70-90%.

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

TABLE 14.

SUMMARY OF RECOMMENDED TEST CONDITIONS FOR
Champia parvula SEXUAL REPRODUCTION TEST

1.	Test type:	Static
2.	Salinity:	30ppt ±2ppt
3.	Temperature:	22-24•C
4.	Photoperiod:	16 h light, 8 h dark
5.	Light quality:	Cool white fluorescent lights
6.	Light intensity:	100 $\mu E/m^2/s$ (500 ft.c.)
7.	Test vessel size:	200 ml polystyrene cups, or 250 ml Erlenmeyer flasks
8.	Test solution volume:	100ml
9.	Dilution water:	30 parts per thousand salinity natural seawater, or a combination of 50% (30 part per thousand salinity) natural seawater and 50% (30 part per thousand) salinity artificial seawater
10.	Dilution factor:	0.3 or 0.5
11.	Number of dilutions:	At least 5 and a control
12.	Number of replicate Chambers per treatment:	4 (minimum of 3)
13.	Number of organisms per test chamber:	5 female branch tips and 1 male plant
14.	Test duration:	2-day exposure to effluent, followed by 5 to 7 day recovery period in control medium for cystocarp development

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

15. Effects measured:	Reduction in cystocarp production compared to controls
16. Test acceptability:	Control survival of 80% or greater, control average cystocarp production of 10 or greater per plant. (NOTE: plants fragmenting in lower concentrations may indicate undue stress)

See Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, EPA/600/4-85/013, March 1985.

APPENDIX C

HEALTH AND SAFETY PROCEDURES

HEALTH AND SAFETY PROCEDURES[1]

1.0 GENERAL PRECAUTIONS

1.1 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through the skin, and asphyxiation due to lack of oxygen or the presence of noxious gases.

1.2 Prior to sample collection and laboratory work, personnel should determine that all necessary safety equipment and materials have been obtained and are in good condition.

2.0 SAFETY EQUIPMENT

2.1 Personal Safety Gear

Personnel should use safety equipment as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes. Plastic netting on glass beakers, flasks, and other glassware minimizes breakage and subsequent shattering of the glass.

2.2 Laboratory Safety Equipment

Each laboratory (including mobile laboratories) should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, and eye fountains.

3.0 GENERAL LABORATORY AND FIELD OPERATIONS

3.1 Work with effluents should be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see safety manuals listed in Paragraph 5.0). It is recommended that personnel collecting samples and performing toxicity tests should not work alone.

3.2 Because the chemical composition of effluents is usually only poorly known, they should be regarded as potential health hazards and exposure to them should be minimized.

3.3 It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

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[1]Adapted from "Short-term Methods for Estimating Chronic Toxicity of Effluents & Receiving Waters for Marine and Estuarine Organisms," EPA, May 1988.

3.4 All containers should be adequately labeled to indicate their contents.

3.5 Good housekeeping contributes to safety and reliable results.

3.6 Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.7 Mobile laboratories should be properly grounded to protect against electrical shock.

4.0 DISEASE PREVENTION

4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against tetanus, typhoid fever, and polio.

5.0 SAFETY MANUALS

5.1 For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, check with the permittee and consult general safety manuals, including the USEPA Occupational Health and Safety Manual (1977), and Health and Safety for Toxicity Testing by D.B. Walters and C.W. Jameson, Butterworth Publishers, Woburn, Massachusetts (1984).

6.0 WASTE DISPOSAL

6.1 Wastes generated during toxicity testing must be properly handled and disposed of in an appropriate manner. Each testing facility will have its own waste disposal requirements based on local, State, and Federal rules and regulations. It is extremely important that these be known, understood, and complied with by all persons responsible for performing toxicity tests.