8. TOXICITY

Contents Page

List of Tables

Related Websites

Whole Effluent Toxicity (WET) Methods: http://www.epa.gov/waterscience/WET
Office of Wastewater Management (OWM) Homepage: http://www.epa.gov/owm

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8. A. Objectives

By definition, toxicity is a characteristic of a substance (or group of substances) that causes adverse effects in organisms. Adverse effects include an increased rate of morbidity (the rate of occurrence of disease) and mortality (the rate of occurrence of death), as well as those effects that limit an organism's ability to survive in nature, such as impaired reproductive ability or growth. 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 effects at a lower concentration.

Whole Effluent Toxicity (WET) is a National Pollutant Discharge Elimination System (NPDES) permit parameter designed to evaluate the toxicity of the entire wastestream as opposed to just individual pollutants. The WET testing may be either performed or evaluated as part of one of five NPDES inspections:

- Compliance Evaluation Inspection (CEI)
- Compliance Sampling Inspection (CSI)
- Performance Audit Inspection (PAI)
- Toxics Sampling Inspection (XSI)
- Compliance Biomonitoring Inspection (CBI).

In addition, the toxicity of a municipal treatment plant effluent should be considered as part of the Pretreatment Compliance Inspection (PCI), especially if unacceptable levels of toxicity have been demonstrated and the cause of the toxicity has been investigated and found to be from industrial or commercial dischargers contributing to the system.

Methods manuals for Whole Effluent Toxicity testing can be accessed at:

http://www.epa.gov/waterscience/WET.

The inspector should understand the permittee's WET testing requirements so that the appropriate objectives can be met:

- Assess compliance with NPDES permit conditions
- Determine compliance with State water quality standards
- Consider overall Lab WET test performance (reference toxicants and other WET QA/QC requirements)
- Evaluate quality of self-monitoring data
- Assess adequacy of self-monitoring procedures
- Document presence or absence of toxic conditions
- Identify need to perform Toxicity Reduction Evaluation (TRE) and/or a Toxicity Identification Evaluation (TIE)
- Develop permit limits for WET, if appropriate

WET test reviews performed as part of a routine facility inspection are cursory. The intent is to quickly ascertain if the facility is following their permit requirements and, secondarily, to see if there are any obvious problems with reporting or lab performance. The following checklist provides some of the more obvious and quickly determined issues that can be addressed during a facility inspection.

- Does the facility have a copy of its NPDES permit readily available? (Although the inspector should bring a copy in the event the permittee cannot find his).
- Check the permit for the WET testing frequency and any special conditions related to WET testing, including whether a testing frequency decrease is authorized.
- Are *all* test reports for WET tests performed over the last three years available for review?
- Are the test reports complete (e.g., bench data sheets for chemicals and test organisms, chain of custody tags, statistical analyses, etc.)?
- Was the correct type test performed?
- Did effluent samples contain any measurable chlorine, or > 10 mg/l ammonia?
- Was the test initiated within 36 hours of the first test sample being grabbed or removed from the compositor? This can be verified by checking dates and times on chain-of-custody tags and bench sheets.
- Did the lab or permittee make any judgement decisions beyond their authority?
- Were there any aberrations in the test?
- Were the test results reported correctly to the permittee and on the DMR?
- Was the test invalid due to poor control performance?
- If the test was declared invalid, was a retest performed and reported?

In the case of a PAI, the laboratory performing the WET tests is evaluated, as well as the NPDES permittee. This type of inspection requires more extensive information than is presented in this section. The inspector is therefore referred to the Environmental Protection Agency's (EPA) *Manual for the Evaluation of Laboratories Performing Aquatic Toxicity Tests* (EPA-600/4-90/031) for the protocol to perform a PAI.

8. B. Requirements of WET Testing

Types of WET Testing

WET tests are techniques to determine the toxicity of a permittee's discharge or effluent by measuring the responses of organisms to a set of multi-concentration solutions of the effluent and dilution water. The WET test methods, as revised November, 2002, are specified in 40 *CFR* Part 136 and described in the WET methods manuals that can be accessed at http://www.epa.gov/waterscience/WET. Test designs may vary in number of organisms used, duration (acute or chronic) or in the way in which the effluent contacts the organism (flow-through, static, static renewal), depending on suspected toxicants present and how the results are to be used. Range finding (screening) tests normally use few organisms and a single effluent concentration. However, WET testing is usually performed as a definitive testing.

In a definitive test, several groups (replicates) of organisms are exposed for a predetermined length of time to a set of multi-concentration solutions of effluent and dilution water. The tests consist of a control and a minimum of five effluent concentrations, with four replicates of each dilution. See the WET methods manuals for more details. The response of each organism in each test concentration is observed and recorded, and the number of responses is analyzed in relation to the concentrations of effluent to which the organisms were exposed.

WET testing may be performed as either acute or chronic tests. The terms acute and chronic refer to the length of time that the organisms are exposed to the toxicant. The duration of the tests should be specified in the NPDES permit. Generally, acute tests measure short-term effects with impacts usually resulting in death or extreme physiological disorder. A response observed in 96 hours or less typically is considered acute. Chronic tests involve a stimulus that lingers or continues for a relatively long period, often one-tenth of a lifespan or more. Chronic should be considered a relative term depending on the lifespan of an organism. Typically most WET chronic tests run for seven days. Acute effects result in death. A chronic effect may result in death, stunted growth, or reduced reproductive rates.

Common test responses indicating the presence of toxic conditions include:

- Death — Increase in number of organisms killed by a test solution when compared to the control
- Growth — Measurement of reduction in growth compared to the control (including mean weight of an organism)
- Reproduction — Measurement of reduction in reproductive rates compared to the control
- Terata — Increase in number of gross abnormalities shown in early life stages compared to the control.

WET tests are also described according to the way in which organisms are physically exposed to test solutions. The terms flow-through, static renewal, and static are most commonly used to describe the test design type. In a flow-through test, effluent and dilution water are

mechanically renewed continuously. 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 less sophisticated equipment. The method of test design type should be specified in the NPDES permit for the acute test methods. The selection of test design type for the chronic test methods is pre-described in the test methods.

WET Test Components

The following discussions pertain primarily to issues in a lab audit.

WET tests consist of a number of components, as shown below:

- Effluent
- Dilution water
- Test apparatus
- Test organisms
- Reference toxicants
- Test results.

In simple terms, effluent and dilution water are combined in the test system with test organisms to produce test results. Each component including food items must be of a specific quality for successful toxicity testing. It is the inspector's job to determine (insofar as possible) from the information available, that the test components adhere to the standards specified in the NPDES permit or accepted reference method (e.g., EPA's WET methods at 40 *CFR* Part 136). Review of the permittee's sampling logbook, chain-of-custody forms, and contract lab reports should provide most of the information necessary to assess the quality of the test components.

Each component has specific requirements (e.g., sample location for the effluent, sample holding time, dilution water constituents, choice of test apparatus materials). Accurate and reproducible test results can only be expected when the critical test components are handled properly. It is, therefore, very important to understand the relationships between these test components and the critical factors that determine the acceptability of each from a quality assurance standpoint. Critical factors that would likely be encountered during a NPDES inspection are described in the following sections.

Effluent

Effluent sampling strategy will usually be specified in the NPDES permit. Effluent samples must be representative of the entire discharge and free of contamination from other sources. Samples collected for off-site toxicity testing are to be chilled to 0°-6°C during or immediately after collection, and shipped iced to the performing laboratory.

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 periods ranging from 24 to 36 hours. This volume will depend on the type of test being conducted and the number of dilutions being run. For static renewal 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 dissolved oxygen (DO), pH, salinity, and other chemical analyses without contamination of the test dilutions. This volume will depend on the type of test being conducted and dilutions being run. Table 8-1 provides guidance as to representative sampling strategies for various situations. For some volatile toxicants that are acutely toxic (e.g., chlorine) standard composite sampling does not yield an effluent sample that is representative of the actual discharge due to volatilization of chlorine during sampling, shipping and holding. On-site flow-through testing would yield more appropriate test results where, considering available dilution, the effluent contains measurable amounts of chlorine.

Samples for onsite tests should be used immediately when practical, but must be used within 36 hours of collection. It is usually 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. Note: hand-delivered samples used on the same day of collection do not need to be cooled at 0°-6°C prior to test initiation.

Samples to be used for offsite tests should be iced for shipment and refrigerated (0°-6°C) upon 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 longer period of time to complete the test. In no case should any preservative be added to samples or chemical disinfection performed prior to being tested for toxicity, nor should samples be dechlorinated unless the permit specifically allows for sample dechlorination.

Dilution Water

The choice of dilution water is generally specified in the NPDES permit and depends on the purpose of the toxicity test. Synthetic dilution water is used to evaluate the inherent toxicity of the effluent. Dilution water from the receiving stream or a nontoxic equivalent is used to test for interactions after discharge. Receiving waters, synthetic waters, or synthetic waters adjusted to approximate receiving water characteristics may be used for dilution water, provided that the water meets the qualifications for an acceptable dilution water. Under no circumstances should the dilution water cause any toxic responses in test organisms. A lack of toxic responses in control organisms is evidence of the suitability of the dilution water. Control organisms should have less than or equal to 10 percent mortality in acute tests and less than or equal to 20 percent mortality for chronic tests. EPA manuals describe various techniques for the preparation of synthetic dilution water which may be necessary to use if the natural receiving water exhibits unacceptable levels of toxicity.

Dilution water obtained from receiving waters should be immediately used for testing. If it will not be used within 24 hours, it should be refrigerated (0°-6°C) as soon as it is collected. In any case, the receiving water should be used within 36 hours of collection. So that no appreciable change in toxic characteristics occurs before testing, the lapsed time (holding time) from sample collection to first use of the sample in test initiation must not exceed 36 hours unless a

variance has been granted. If holding is necessary, the samples must be stored under strict conditions (temperatures for WET samples as 0°-6°C). The location from which the dilution water was obtained should be noted in the permittee's sampling log. It should be upstream and out of the influence of the outfall. The location should be free of other sources of contamination (e.g., other outfalls).

Table 8-1

Recommended Sampling Strategies for Continuous and Intermittent Discharges for Flow-Through, Static Renewal, and Static Toxicity Tests*

CONTINUOUS DISCHARGE

TEST TYPE	CHRONIC	ACUTE Retention Time < 14 Days	ACUTE Retention Time >14 Days
Flow-through**	-	Two Grab samples daily (early a.m. and late p.m.)	One grab sample daily
Static renewal	3x 24-Hour composite samples, every other day	Four separate grab samples each day for four concurrent tests	One grad sample daily
Static	Single 24-Hour composite sample on first day	Four separate grab samples on first day for four concurrent tests	One grab sample on first day

* Sampling requirements should be clearly specified in the permit

* For flow-through tests, it is always preferable to pump directly to the dilutor

INTERMITTENT DISCHARGE

TEST TYPE	CHRONIC	ACUTE Continuous Discharge During 1 or 2 Adjacent 8-Hour Shifts	ACUTE Discharge From Batch Treatment	ACUTE Discharge to Estuary on Outgoing Tide
Flow-through	-	One grab sample midway through shifts daily	One grab sample of discharge daily	One grab sample of discharge daily
Static renewal	3x 24-Hour composite samples collected for duration of discharge unless discharge ceases	One grab sample midway through shifts on first day	One grab sample of discharge daily	One grab sample of discharge daily
Static	Composite sample collected for duration of discharge, first day	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

Test System

WET tests may be performed in a fixed or mobile laboratory. Depending on the scope of the program, facilities may include equipment for rearing, holding, and acclimating test organisms. Temperature control is achieved using circulating water baths, heat exchangers, or environmental chambers. Appropriate dilution water may be groundwater, surface water, reconstituted water, or dechlorinated tap water. Holding, acclimation, and dilution water should be temperature controlled and aerated whenever possible. Air used for aeration must be free of oil and fumes; filters to remove oil in air are desirable. Test facilities must be well-ventilated and free of fumes. During holding, acclimating, and testing, test organisms should be shielded from external disturbances. Reference toxicants should be properly stored in a closed area separate from the WET testing areas.

Any materials that come into contact with either effluent or dilution water must not release, absorb, or adsorb toxicants. A number of different choices for test equipment are 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 soldered. Other specialized containers of nitex or teflon are also acceptable. Tanks for storing effluents and dilution water may also be made of fiberglass. All containers or tubes made of these materials are reusable with appropriate cleaning (see below).

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.

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) nitric or hydrochloric acid, rinsed in tap water, and then rinsed with dilution water before use.

Glassware and stainless steel components that must be reused should be soaked in detergent and scrubbed (or washed in a laboratory dishwasher), 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 be neutralized in sodium bicarbonate before use.

Test Organisms

Organisms used for toxicity testing are limited to certain species for which there are established testing protocols (EPA 40 *CFR* Part 136). Species commonly used in biomonitoring include daphnids, mysids, fathead minnows, silversides, and algae. 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. Therefore, it is important that these factors comply with accepted test method procedures. Test conditions for various types of tests and organisms can be accessed at: http://www.epa.gov/waterscience/WET.

The inspector should ascertain, as closely as possible, that the following procedures are being observed:

- The correct organisms must be utilized in the test (most often as specified in the NPDES permit). "Wild" organisms are rarely appropriate in WET testing. Test organisms used in toxicity testing must be of known history, free of disease, and acclimated to test conditions. Culture information should be recorded. Test organisms must also be of the appropriate age. The appropriate number of organisms must be used in each test vessel.

- Test organisms should be fed according to the requirements for the particular type of test. When feeding is necessary for mysid or fish tests, excess food should be removed daily by aspirating with a pipette, to avoid problems such as food buildup leading to excessive oxygen demand.

- A daily log (that is a daily bench sheet for each test being performed) should be kept by the lab of feeding, reproduction, growth, mortality, and any abnormal behavioral observations.

- The following procedures must be adhered to (by the contract laboratory) for holding test organisms:

  - Test organisms purchased may be used to start mass cultures. However, if the organisms are to be used for testing then they must be no more than 48 hours old (if fish, purchased and shipped) and must be <24 hours old (fish, if not shipped, and freshwater invertebrates) at the start of the test. Freshwater invertebrates used in a test must also have all been released within an 8 hour period, to ensure reproductive performance is not impacted.

  - Maintain DO levels above 4 mg/L for warm water species and above 6 mg/L for cold water species.

- The laboratory should record the source of test organisms (hatchery, in-house, or elsewhere), as well as holding conditions (temperature, dissolved oxygen).

- Test organisms should be handled as little as possible to minimize stress:

  - Dip nets should be used for large organisms

- Pipettes should be used for transferring small organisms such as daphnids and midge larvae.

Reference Toxicants

Reference toxicants are used to evaluate the health and sensitivity of test organisms over time and for documenting initial and ongoing laboratory performance. A laboratory performs a definitive toxicity test with a reference toxicant at least once per month using each toxicity test method conducted in that month. The monthly results are plotted on a control chart to track trends in organism health or sensitivity.

EPA does not require the use of specific reference toxicants and does not set required acceptance ranges for reference toxicant testing. Testing laboratories must perform at least one acceptable reference toxicant test per month for each type of toxicity test method conducted in that month regardless of the source of test organisms. If a test method is conducted only monthly, or less frequently, a reference toxicant test must be performed concurrently with each effluent toxicity test to document ongoing laboratory performance and assess organism sensitivity and consistency when organisms are cultured in-house. When organisms are obtained from external suppliers, concurrent reference toxicant tests must be performed with each effluent sample, unless the test organism supplier provides control chart data from at least the past five months of reference toxicant testing, which will assess organism sensitivity and health. The method manuals require a laboratory to obtain consistent, precise results with reference toxicant toxicity tests with effluents under the NPDES permits.

An attempt should be made to match the type of reference toxicant used (e.g., metal or chlorinated organic) to the major pollutant in the wastewater tested. Reference toxicant data must be included with the contract lab report.

Reference toxicant test results should not be used as *de facto* criteria for rejection of individual effluent or receiving water tests. The methods manuals provide guidance for what to do when more than 1 reference test in 20 reference toxicant tests falls outside of control chart limits, or when a reference toxicant test result falls “well” outside of control limits. The laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month.

Conduct of the Test(s)

Test methods should be used by analysts experienced in the use or conduct of aquatic tests and the interpretation of data from aquatic toxicity testing. Test conditions should be those as specified in the summary of test condition tables provided for each method. Physical and chemical measurements taken during the test (e.g., temperature, pH, and DO) must be conducted at a minimum as specified in the method manuals. The test methods should follow the procedures as described in each test method section of the manual following the table of recommended test conditions. Test organisms should be obtained and added according to the guidance in any specific method.

Recordkeeping and Data Reporting

Proper recordkeeping is essential to an effective program. Chain-of-custody (COC) procedures should consistently be used to document sample transfer. Hand-written entries on bench sheets and COC tags must generally be clear and legible. The permittee should maintain a sample log containing information as to the date, time, and type of sample taken as well as the sampler's name. Unusual conditions should be noted. When evaluating the contract lab's data reporting, the inspector should verify that the following are included:

- Summary of test results, description of test conditions, material tested, and other data for quality assurance.
- Methods used for all analyses. The method title, method number and method source should be provided in the laboratory standard operating procedure (SOP) and test report. Tests must be conducted as stated in SOP and laboratory should verify test was conducted according to SOP.
- Date and time test started; date and time test terminated, type and volume of test chambers, volume of solution used per chamber, number of organisms per test chamber, number of replicate test chambers per treatment.
- The test temperature (mean and range), details of whether test was aerated or not, feeding frequency, and amount and type of food, any pH control measures taken.
- Any deviation from standard test methods. The test endpoint(s), and any deviation(s) from method must be clearly noted.
- The reference toxicity results for tests conducted for the test period with specific test details to verify species, temperature, and dilution water used in reference toxicant test.
- Any acclimation of test organisms (temperature mean and range) and the reason(s) for acclimation.
- Any other relevant information.

It is important that the contract lab to have a copy of the permittee's NPDES permit, including any modifications. By having a copy of the permit, the lab can better ensure that proper test procedures are being followed.

Any deviations from specifications should be documented and described in the data report by the testing laboratory. For WET test data submitted under NPDES permits, all required test conditions must be met or the test is considered invalid and must be repeated with a newly collected sample. Deviations from recommended test conditions must be evaluated on a case-by-case basis to determine the validity of test results. Deviations from recommended test conditions may or may not invalidate a test result depending on the degree of the departure and the objective of the test. Consideration of the degree of the deviation and the potential or observed impact of the deviation on the test result before rejecting or accepting a test result is valid. For example, if dissolved oxygen is measured below 4.0 mg/L in one test chamber, the reviewer should consider whether any observed mortality in that test chamber corresponded with the drop in dissolved oxygen. Whereas slight deviations in test conditions may not

invalidate an individual test result, test condition deviations that continue to occur frequently in a given laboratory may indicated the need for improved quality control in that laboratory.

Data for each test should be provided as the raw toxicity data in tabular form, including daily records of affected organisms in each concentration (including controls) and replicate, and in graphical form (plots of toxicity data) and include a table of LC_{50}s, NOECs, IC_{25}, IC_{50}, etc. (as required in the applicable NPDES permit). Records should indicate statistical methods used to calculate endpoints, and have a summary table of physical and chemical data. Testing laboratories should maintain quality assurance/quality control (QA/QC) control charts for percent minimum significant difference (PSMD) along with the statistical endpoints such as NOEC, LC50, EC25. Testing laboratories should regularly plot the individual raw test data and the average treatment responses to examine possible causes of excessive variability. For more information on possible contributing factors to WET variability and recommendation for reducing it, see sec 7.3 *of Understanding and Accounting for Method variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination System Program*, U.S. EPA, 2000. EPA/833/R-00/003.

The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. All WET test results (from multi-concentration tests) reported under the NPDES program should be reviewed and reported according to USEPA guidance on the evaluation of concentration-response relationships (USEPA, 2000a). This guidance provides review steps for 10 different concentration-response patterns that may be encountered in WET test data. Based on the review, the guidance provides one of three determinations: that calculated effect concentrations are reliable and should be reported, that calculated effect concentrations are anomalous and should be explained, or that the test was inconclusive and the test should be repeated with a newly collected sample. It should be noted that the determination of a valid concentration-response relationship is not always clear cut. Data from some tests may suggest consultation with professional toxicologists and/or regulatory officials. Tests that exhibit unexpected concentration-response relationships also may indicate a need for further investigation and possible retesting. Each test must be reviewed to ensure that the test acceptability requirements have been met and that the data from the calculated test results are interpreted appropriately (USEPA, 2002a). Test review should include reviewing reference toxicant testing and the within-test variability should be reviewed. EPA's preferred method of data analysis is point estimation, but when NPDES permit require sublethal hypothesis testing endpoints, the within-in test variability must be reviewed and variability criteria applied. When tests are used for non-regulatory purposes, the variability is not required.

In addition to reviewing the concentration-response relationship, the within-test variability of individual tests should be reviewed. When NPDES permits require sublethal hypothesis testing endpoints (e.g., reproduction for the *Ceriodaphnia dubia* test), within-test variability must be reviewed and variability criteria must be applied as described in the chapter on “Report Preparation and Test Review” of each manual. Compare the PMSD measured in the test with the PMSD bounds listed in the report chapter. When the methods are used for non-regulatory purposes, the variability criteria are recommended but are not required, and their use (or the use of alternative variability criteria) may depend upon the intended uses of the test results and the requirements of any applicable data quality objectives and quality assurance plan.

Within-test variability is measured as the percent minimum significant difference (PMSD) and must be calculated and compared to the upper bounds that are established for test PMSDs. Tests conducted under NPDES permits that fail to meet this variability criteria and that show “no toxicity” at the permitted receiving water concentration (i.e., no significant difference from the

control at the receiving water concentration or above) are considered invalid and must be repeated on a newly collected sample. Lower bounds on the PMSD are also applied, such that test concentrations are not considered toxic (i.e., significantly different from the control) if the relative difference from the control is less than the lower PMSD bound.

To avoid penalizing laboratories that achieve unusually high precision, lower PMSD bounds are applied when a hypothesis test result (e.g., no observed effect concentration (NOEC) or lowest observed effect concentration (LOEC)) is reported. Lower PMSD bounds are based on the 10^{th} percentiles of national PMSD data. The 10^{th} percentile PMSD represents a practical limit to the sensitivity of the test method because few laboratories are able to achieve such precision on a regular basis and most do not achieve it even occasionally. In determining hypothesis test results, a test concentration is not considered toxic if the relative difference from the control is less than the lower PMSD bounds. See *Understanding and Accounting for Method variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination System Program*, U.S. EPA, 2000. EPA/833/R-00/003 for specific examples of implementing lower PMSD bounds. To reduce within-test variability and to increase statistical sensitivity when test endpoints are expressed using hypothesis testing rather than the preferred point estimation techniques, variability criteria must be applied during test review when NPDES permits require sublethal hypothesis testing endpoints NOEC or LOEC and the effluent is determined to have no toxicity at the permitted receiving water concentration.

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8. C. Analysis of Results

Each test manual has specified test acceptability criteria (e.g., minimum control survival) that must be achieved in order to have an acceptable test result. See the summary of test conditions and TAC for the specific test method section of the manual. In general, the valid interpretation of test results requires that control organisms meet minimum criteria for survival, growth, and/or reproduction.

Mortality in controls must 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. For chronic tests, control organisms also must meet minimum requirements for growth and reproduction contained in the methods. Tests not meeting the test control acceptability criteria (TAC) to achieve survival, growth, or reproduction are not valid. When using dual controls, the dilution water control should be used for determining the acceptability of the test and for comparisons with the tested effluent.

Each test manual has specified acceptable ranges of test conditions that are to be met, such as temperature, dissolved oxygen concentration, salinity, pH, light intensity and duration of photoperiod, organism loading (numbers or weight per volume), feeding, and cleaning procedures. Tests not meeting the other test conditions in the Summary of Test Conditions and TAC for the specific test method should be reviewed with caution and referred to the regional biologist. For each parameter discussed in these tables, the parameter is either recommended (i.e., must do) or required (i.e., should do). For example, the chronic *Ceriodaphnia dubia* test type is static renewal and specified as required. Meaning the test type for this test method must be static renewal. For WET test data submitted under NPDES permits, all required test conditions must be met or the test is considered invalid and must be repeated with a newly collected sample. The inspector should review the EPA methods manual for a more extensive discussion of each of these factors. The EPA methods manuals for Whole Effluent Toxicity testing can be accessed at: http://www.epa.gov/waterscience/WET.

After a test has met the required TAC and test conditions, the next step is data review (see chapter on “Report Preparation and Test Review” of each manual). Test review should be conducted on each test by both the testing laboratory and the regulatory authority.

The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. EPA provides guidance on reviewing concentration-response relationships (USEPA, 2000). Test results that do not meet the expected pattern may be determined to be reliable, anomalous, or inconclusive.

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 abnormalities occur (outside of experimental error), the results and test conditions should be reviewed by the regional biologist or NPDES toxicologist. It should be recognized, however, that often there will be minor variations in test results. For example, *Ceriodaphnia dubia* reproduction may be higher at intermediate concentrations that 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 - see *Understanding and Accounting for Method variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination System Program*, U.S. EPA, 2000. EPA/833/R-00/003 for specific examples.

The test results need to be expressed such that compliance with the permittee's WET limits can be determined. For the NPDES Permit Program, the point estimation techniques are the preferred statistical methods in calculating end points for effluent toxicity tests.

The following definitions may help the inspector to interpret the results:

- 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. 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} (for effect concentration) 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 the EC_{50} and the LC_{50} when the response is a surrogate for death.

- The No Observed Effect Concentration (NOEC) is the highest tested concentration at which the organisms' responses are not statistically different from the control organisms' responses. The NOEC [like the Lowest Observed Effect Concentration (LOEC) and Chronic Value (ChV) defined in the following paragraph] is normally determined only for chronic tests.

- The LOEC is the lowest tested concentration at which organisms' responses are statistically different from controls.

- The ChV is the calculated geometric mean of the NOEC and LOEC (the square root of the product of the NOEC and LOEC).

- The Inhibition Concentration (IC_{25}) 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.

- The percent response at a criterion concentration is reported. For example, the permit or standard may prohibit toxicity at 100 percent effluent or less. In this case, the observed percent response at 100 percent effluent would be reported.

- The response may be reported in Toxic Units (TU), either as Acute TUa or Chronic TUc.

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 indicates higher toxicity than test results at higher percentages of effluent. TUs are defined as $100/LC_{50}$ for acute or 100/NOEC for chronic, with the LC_{50} or NOEC expressed as percent effluent. An effluent with an LC_{50} of 50% has an acute toxicity of 2.0 acute toxic units (100/50 = 2). Similarly, an effluent with a NOEC of 25% effluent has a chronic toxicity of 4 chronic toxic units (100/25). 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 the magnitude of a TU indicates the degree of toxicity. Therefore, an effluent with a TUa of 4 is twice as toxic as an effluent with a TUa of 2. EPA's Technical Support Document for Water Quality-based Toxics Control (EPA/505-2-90-01, 1991) provides a more extensice discussion of the application of toxic units and the relevance to NPDES permits. EPA's Technical Support Document (TSD, 1991) provides a more extensive discussion of the application of TU's and their relevance in an NPDES permit.

In addition to reviewing the concentration-response relationship, the within-test variability of individual tests should be reviewed. When NPDES permits require sublethal hypothesis testing endpoints (e.g., reproduction for the *Ceriodaphnia dubia* test), within-test variability must be reviewed and variability criteria must be applied as described in the chapter on "Report Preparation and Test Review" of each manual. Compare the PMSD mesaured in the test with the PMSD bounds listed in the report chapter. When the methods are used for non-regulatory purposes, the variability criteria are recommended but are not required, and their use (or the use of alternative variability criteria) may depend upon the intended uses of the test results and the requirements of any applicable data quality objectives and quality assurance plan.

See *Understanding and Accounting for Method variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination System Program*, U.S. EPA, 2000. EPA/833/R-00/003 for specific examples.

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8. D. References

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