

Hydrosphere Research



# Understanding Aquatic Toxicity Testing

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# Understanding Aquatic Toxicity Testing

## Introduction

This information packet has been prepared as a resource for Hydrosphere Research NPDES permit holders required to perform effluent toxicity testing or are utilizing toxicity testing as part of a toxicity reduction program. The document explains many of the concepts and terminology used by a regulatory agency regarding toxicity testing. By carefully reading this information it is hoped that further communication with either your own environmental staff or an outside consultant will be made more productive. This document may be found at <http://www.hydrosphere.net/Understanding-Tox.pdf>. It is critical that you read and understand your permit requirement and share that information with the Hydrosphere Research staff. Good lines of communication are essential to ensure that appropriate testing is scheduled regardless of the circumstances requiring such testing.

Toxicity refers to the potential for a substance to produce an adverse or harmful effect on a living organism. A toxicant is an agent (e.g., whole effluent discharge) that can produce an adverse effect in a biological system, seriously damaging its structure or function or causing death. The adverse response may be defined in terms of a measurement that is outside the "normal" range for healthy organisms, such as abnormal mortality, reproduction or growth.

Toxicity tests determine the level of toxicity, if any, present in an effluent and the duration of exposure required for the toxicity to be expressed as adverse effects. Organisms are exposed in test chambers to various concentrations of the effluent. The criteria for effects, such as mortality and reproduction, are evaluated by comparing those organisms exposed to different dilutions of the effluent with those organisms (controls) exposed only to nontoxic dilution water.

Acute effects are those that occur rapidly as a result of short-term exposure. Exposure is considered relative to the organism's life span. The most commonly measured acute effect in aquatic organisms is death. Chronic effects occur when an effluent or toxicant produces adverse effects as a result of a repeated or long-term exposure. Chronic effects include lethal and sublethal responses (such as abnormal growth and/or reproduction).

Statistical analyses and mathematical modeling summarize the data collected during a toxicity test. The specific application of these routines may be quite simple or extremely complex. The final analysis (after these statistics have been performed) however, is easily understood. All statistical routines are specifically defined for each procedure. It is not necessary to completely understand all of the analyses performed by a laboratory in order to utilize data produced by toxicity testing. This document includes an overview of these data interpretations.






In measuring the acute toxicity of an effluent, the objective is to measure a range of effluent concentrations or one specific concentration that produces a readily observable and quantifiable response. The quantifiable response most often observed is mortality, which is then used to calculate an LC50 value or determine if significant acute mortality is occurring. The LC50 is the concentration estimated to cause mortality in 50% of the test population over a specified time period. Application factors may be applied to a measured LC50 to predict the concentration of effluent that may have no adverse impacts over an extended duration.

Rather than using an acute test with an application factor to evaluate chronic toxicity, it is possible to directly measure chronic impacts with a more sophisticated test procedure. These chronic tests are more difficult to perform but eliminate use of an artificial application factor. The chronic test measures both sublethal and lethal effects over a longer test duration and measures responses during a sensitive period of the organism's life cycle.

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### General Considerations

The toxicity tests that the Hydrosphere Research utilize and that NPDES permittees are required to conduct are controlled laboratory experiments in which effluent concentration is the primary variable by which the response is evaluated. These tests are standardized to maximize comparability and reproducibility. Toxicity test protocols typically specify the exposure of test organisms to fixed concentrations of whole effluent for a defined time period. Species selection (test organism) is one element that defines a specific toxicity testing procedure. Test species are selected based upon the ease of laboratory culture, the availability of adequate background information such as its physiology, genetics, and behavior and sensitivity to a wide range of toxicants. The test species is usually one of the following: the water fleas *Ceriodaphnia dubia*, the Fathead Minnow, *Pimephales promelas*, the Bannerfin Shiner, *Cyprinella leedsi*, the Mysid Shrimp, *Mysidopsis bahia*, or the Silverside Minnow, *Menidia beryllina*. Other species may be utilized to address a specific concern. These tests are typically static, meaning the organisms are maintained in the original test solutions for the duration of the test.

	Freshwater	Saltwater
Invertebrates	 Water Flea	 Mysid Shrimp
Vertebrates	 Fathead Minnow	 Silverside Minnow
	 Bannerfin Shiner	

All of the toxicity tests include a control (or untreated sample) to ensure that the effects observed are associated with or are attributable to exposure to the test material. This provides the baseline for interpreting the test results by identifying unwanted variables.

The effluent samples are prepared for testing by being thoroughly mixed, allowed to reach standard test temperature, and aerated if dissolved oxygen (DO) is below 4 mg/L. Total residual chlorine is measured. The effluent is then diluted with control water, typically to five concentrations (with the appropriate number of replicates) from 0 to 100% effluent. The test vessels are filled with the appropriate volume of test solution. Test organisms are transferred to test chambers in a random manner. Initial DO and pH are measured in separate vessels of dilution and effluent solutions. The test is incubated at 25°C with a 16:8 hour light:dark cycle. Mortality of the test organisms is recorded after the defined test period along with final pH, dissolved oxygen, and temperature.

### Sample Collection

Prior to collecting an effluent sample and performing a toxicity test, sampling glassware and stainless steel or Teflon equipment are washed with soap and hot water, then rinsed in nitric acid, acetone, and distilled/deionized water to remove toxicants and contaminants. Plastic containers and equipment may be used on a one-time or disposable basis, or dedicated to use with a particular effluent. The effluent sample used in the static tests is collected below chlorination as a grab or 24-hour composite (depending on permit requirements). The sample must be collected and stored with an amount of ice sufficient to maintain its temperature between 0° and 6°C until receipt at the laboratory. Hydrosphere Research is required to measure sample temperature on receipt of all samples. Should this temperature exceed allowable standards, the sample does not qualify for the performance of valid tests and such results will be rejected for use in NPDES compliance determinations. Additionally, the sample is not to be frozen under any circumstances. Frozen samples will be rejected for use in NPDES compliance determinations. Coordination of sampling and sample shipment methods should be discussed with Hydrosphere Research so that these criteria are met.

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### Acute Toxicity Tests

Acute toxicity tests determine whether some concentration of test material or effluent will produce an adverse effect on a group of test organisms during a short-term exposure under controlled conditions. Experimentally, a 50% lethal response (concentration at which 50% of the test organisms die) is the most reproducible measure of acute toxicity. When the median lethal concentration (LC50) is calculated, the 95% confidence limits associated with that value are also reported. Acute toxicity tests may have duration of 24, 48, or 96 hours.

An LC50 or concentration of effluent lethal to 50% of the test organisms over the test period is calculated from the mortality data using one of the several methods, preferably the Probit Method or Spearman-Kärber Method, as described in the EPA acute testing protocols (EPA/600/4-90/027F).

### Chronic Toxicity Tests

Chronic toxicity tests allow evaluation of adverse effects of an effluent under conditions of long-term exposure. Lengthening the test duration to include one or more complete life cycles or performing the test during a sensitive life stage allows the detection of more subtle adverse effects, such as reduction in growth and reproduction. Evaluation of these effects from long-term exposure to the effluent can provide a direct estimate of the effect threshold of the toxicant. During life cycle tests with several species of fish and invertebrates, certain developmental stages have consistently been shown to be more sensitive than others. Use of shorter tests with the early developmental stages can also predict chronic toxicity. These methods have been developed to provide quicker and less costly ways to measure chronic toxicity to aquatic organisms.

NPDES testing requires a three brood static renewal test using the cladoceran, *Ceriodaphnia dubia*, as the test organism. A static renewal test is one in which the test solutions are renewed periodically by transferring the test organisms to chambers with freshly prepared solutions. The test is initiated with organisms that are less than 24 hours old and born within 8 hours of each other.

The *C. dubia* chronic toxicity test measures both survival and reproduction during the test period. The original neonate (newly born *Ceriodaphnia*) introduced into each test container at the beginning of the test is monitored for survival as well as for the number of offspring it produces. The *P. promelas* chronic toxicity test measures both survival and growth during the test period. The original Fathead Minnow introduced into each test container at the beginning of the test is monitored for survival. At test termination, the weight of the control group of fish is compared to the test exposure groups.

Exposure of the organisms to differing concentrations of effluent can determine the concentration of effluent expected to cause significant mortality, suppression of growth, or suppression of reproduction, as compared to control populations. The endpoints of these multiple concentration tests can often be described by the highest concentration that causes no observed effect or the NOEC (No Observed Effect Concentration).

The statistical comparisons for evaluating the significance of chronic analysis test results are generally performed as outlined in the EPA guidance documents referenced (EPA-600-4-91-002, EPA-600-4-91-003). Statistical significance may be evaluated in part by calculation of Dunnett's T value. The use of this test is discussed in the EPA document. Significant differences in mortality rates are determined by use of the Fisher's Exact Test as discussed in the cited EPA document.

Chronic toxicity analysis quality control parameters for control organisms include average total reproduction, which must equal or exceed 15 offspring per surviving female. Also, mortality greater than 20% in the control population will be considered abnormal, invalidating the test results. Other quality control components of the test include incubating the test chambers for temperature control, maintaining a photoperiod of 16 hours of light and 8 hours of darkness, use of samples within 72 hours of collection and maintenance of samples between 0-6°C during shipping and storage.

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### Reporting Toxicity Test Results

Under most circumstances, toxicity testing results from samples collected prior to the permitted discharge point are not required to be reported. Such samples include pre-chlorination samples, treatment process samples, and industrial process samples. However, any result from a test performed on a sample collected from the permitted discharge point must be reported. This requirement applies even to those facilities that do not have toxicity monitoring in their NPDES permit.

Test results may be rejected due to inappropriate sampling, inadequate control organism survival, or in the case of chronic tests, inadequate control organism reproduction. Hydrosphere Research term such an analysis "invalid." Under these circumstances a follow-up test must be initiated within 30 days of the initial monitoring event.

At times Hydrosphere Research may be aware of QA problems during or immediately following a test that will prevent the data from being accepted. Additionally, a test may be scheduled that cannot be completed due to sample collection or shipment problems. In such cases the analysis should be rescheduled within 30 days of the initial monitoring attempt. If the analysis cannot be rescheduled during the permit defined monitoring month, Hydrosphere Research will draft a letter that explains why the analysis could not be completed during the appropriate monitoring month and specifies the rescheduled date of the analysis. While this letter does not relieve the facility from completing the monitoring, it will help to prevent Notices of Violation for failure to perform the initial monitoring.

### Statistical Significance

Presenting and interpreting acute and chronic toxicity test results requires the use of statistical analysis. Supporting statistics are used to evaluate the level of confidence that may be associated with the test results.

In an acute toxicity test, the primary purpose of the test is generally an estimation of the concentration of the test material or percentage of effluent that is lethal to 50% of the test organisms within a specific length of time. This measure is called an LC50. The LC50 is chosen in most acute toxicity tests because an estimate of the median tolerance (50% kill) for a fixed sample size is most reproducible in this range. The LC50 is statistically estimated because it is unlikely that one of the concentrations selected in the experiment will kill exactly 50% of the exposed test population. A confidence interval for the true LC50 is computed along with this point estimate and asserts with a pre-specified level of confidence (usually 95%) that this interval contains the true LC50. EPA acute toxicity testing protocols (EPA/600/4-90/027F) describe several methods for estimating the LC50 and confidence intervals. Although any of the referenced methods are acceptable, the recommended methods are the Probit and Spearman-Kärber Methods because their LC50 estimates rely on the data in the more stable, central portion of the tolerance distribution.

The chronic toxicity tests determine the effects of whole effluents on the mortality, growth, or reproduction of a species (*C. dubia* or *P. promelas*) for an extended period of time. Mean reproduction, growth, and percent mortality results for the effluent concentration are compared to those for the control by performing statistical tests of significance.

### Summary

Hopefully this document helps explain toxicity testing concepts and terminology. The best way to learn about our laboratory and what goes on here is to visit and see the operations first hand. We invite site visits anytime. Please call toll-free at (866) 375-9004 to arrange a visit.