

Laboratory Guidelines
for Conducting Whole Effluent Toxicity Tests

COLORADO DEPARTMENT OF PUBLIC HEALTH
AND ENVIRONMENT

WATER QUALITY CONTROL DIVISION

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Introduction

This document provides guidance for conducting whole effluent toxicity (AWET®) tests as required in Section 6.9.2 (5) of the Regulations for the Colorado Discharge Permit System (CDPS) (5 CCR 1002-2), and as referenced in the Water Quality Control Division's (Division) policy for aquatic life WET testing. It constitutes guidelines only and is not rulemaking. The basis for toxicity testing in permits is provided in the regulations.

Primary guidance for conducting WET tests is found in *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms - Third Edition* (EPA/600/4-91/002 July 1994), *Methods for Measuring the Acute Toxicity of Effluents and Receiving Water to Freshwater and Marine Organisms - Fourth Edition* (EPA/600/4-90/027F August 1993), or most recent editions, and in the *Environmental Protection Agency (EPA) Region VIII NPDES Whole Effluent Toxics Control Program*.

The Division's guidelines use and supplement the procedures found in the referenced EPA guidance. The information contained in the Division's guidelines is not intended to provide all of the information necessary to conduct WET tests and therefore, those labs conducting these tests should follow the methods in the EPA manuals unless specified otherwise in these guidelines. Specific testing requirements (e.g., type of effluent sample) are provided in each CDPS permit, and are the legally binding requirements.

There could be site-specific (effluent-specific) testing conditions and problems which may require some modification of the methods. The Division will consider such requests for changes of this type. It is essential that any modifications be approved in writing and incorporated in the CDPS permit by the Division before testing begins.

Test Organisms

Required species
for acute testing

Testing will be conducted with the daphnid, *Ceriodaphnia dubia*, and the fathead minnow, *Pimephales promelas*, during each testing cycle. Alternative species listed in the Division's WET Permit Implementation Guidance Document may be allowed on a case by case basis.

Required species
for chronic testing

Testing will be conducted with the daphnid, *Ceriodaphnia dubia*, and the fathead minnow, *Pimephales promelas*, during each testing cycle

Dilution Water

Options (in order
of preference)

(1) Moderately hard reconstituted water.

See the EPA references for recipes. Prepare or use fresh batches or supplies that are less than two weeks old and that have been aged for 24 hours before use.

(2) Reconstituted water similar to receiving water.

Prepare water to match the receiving water hardness, and pH (" 15% hardness as CaCO₃, 300 mg/l maximum hardness; pH "0.2 units). See the EPA references for recipes. Prepare or use fresh batches or supplies that are less than two weeks old and that have been aged for 24 hours before use.

(3) Receiving water.

Collect water upstream of the permittee's wastewater discharge. In the case where a discharge is directly to a dry stream bed or to a stream segment not classified for an aquatic life use, the water in the next downstream segment classified for an aquatic life use shall be used. Where this is impractical then a reconstituted water similar to the effluent may be used. Prepare water to match the effluent water hardness, and pH (" 15%, hardness as CaCO₃, 300 mg/l maximum hardness; pH "0.2 units). Prepare or use fresh batches or supply that are less than two weeks old and that have been aged for 24 hours before use.

Acceptability

When possible, it is preferable to determine the acceptability of receiving water and reconstituted water (similar to receiving water) before testing starts. Controls should show acceptable survival during testing. It is recommended that acceptability of dilution water also be determined by demonstrating that test species will survive, grow and reproduce in it. Periodic checks during the year should be conducted to ensure that it remains acceptable.

Performance control

A performance control of moderately hard reconstituted water is recommended when receiving water or reconstituted water similar to receiving water is used for dilution water, because for some situations these types of dilution water may not demonstrate acceptable control survival. To apply to an individual test, a separate performance control must be used for each test and must run concurrently on the same board or boards with that individual test.

In cases of excess receiving water toxicity, as determined by the Division (see [Quality Assurance](#)), statistical comparisons should be made between the concentrations and a performance control.

Sampling frequency for receiving waters (dilution water option 3)

For acute tests, collect one receiving water grab sample immediately before each WET test. For chronic tests, collect receiving water grab sample(s) upstream of the permittee's wastewater discharge

Effluent

Sampling method

Collect a sample type consistent with the type of sample specified in the permit. The permit should specify a sample type consistent with that considered acceptable for other parameters. Generally this will be a single grab for acutes and an 8-h composite for chronics. Grab samples for chronic tests may be permitted if the permittee demonstrates, to the Division's satisfaction, effluent homogeneity.

Sampling frequency

For acute tests, a minimum of one effluent sample is required to conduct each test. Test dilutions must be replaced after each 24 hours with freshly prepared dilutions of the original effluent sample.

For chronic tests, a minimum of three effluent samples is required to conduct each test. In order to accommodate laboratory testing schedules, two of these samples can be taken at intervals of two days and one at three days or the three samples can be collected at two day intervals. One of the three samples can be used for preparing replacement dilutions for a maximum of three days (72 hours). A recommended collection schedule would provide fresh samples at time 0, 2, and 4 days. If an eighth day is required to complete a *Ceriodaphnia* test, then the last sample can be used to prepare a final set of dilutions

Dechlorination of WET samples for testing

Routine dechlorination of WET samples before testing is not acceptable. Dechlorination of samples may be allowed in those situations where a facility does not yet have dechlorination facilities in place and is on a compliance schedule for chlorine limits and dechlorination facilities are being installed.

Chemical Observations

Dissolved oxygen and pH

Measure at the beginning and at the end ("old and new") of each 24-h exposure period in one randomly selected test container in each concentration and in the control.

Conductivity (or TDS)

Measure at the beginning of each 24-h exposure period in one randomly selected test container in each concentration and control.

Temperature

Temperature is measured at the end of each 24-hr exposure period in at least one test chamber at each test concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test vessels at least at the end of the test to determine the temperature variation in the environmental chamber.

Total alkalinity, total hardness, total chlorine, total ammonia (as N)

Measure once in each batch (sample) of effluent and dilution water before using in the test.

All parameters

Measure new solutions in an excess aliquot rather than in test containers to avoid contamination and measure old solutions after removing test animals from solutions.

Quality Assurance

Acceptable survival

Survival in controls must be at least 90%.

for acute tests

Acceptable survival
growth and
reproduction for chronic
tests

Survival in controls must be at least 80%. Average dry weight per surviving control fathead minnow larvae at the end of the test should equal or exceed 0.25 mg. 60% of the surviving *Ceriodaphnia* in the controls must have had at least three broods, with an average total number of 15 or more offspring per surviving female.

Conditional
Acceptability
Criteria

An individual test may be conditionally acceptable, depending on the degree of the departure, if the temperature, dissolved oxygen, or other specified conditions, except for reproduction, growth or lethality in the control, fall outside specifications. The acceptability of the test will depend on the best professional judgment of the laboratory, permittee, and the Division.

In those situations where a performance control has been conducted concurrently with a test, and acute test lethality exceeds 10% in a reconstituted water similar to receiving water control, this guidance provides for conditional acceptability (validity) where survival in the lowest effluent concentration and in a performance control is 90% or greater.

In those situations where a performance control has been conducted concurrently with a test, and chronic test mortality exceeds 20% in a reconstituted water similar to receiving water control, this guidance provides for conditional acceptability where survival in the lowest effluent concentration and in the performance control is 80% or greater

The Division must be contacted to make these determinations. In these cases, the performance control will be substituted for the purpose of conducting statistical comparisons.

Any deviation from test specifications must be noted on the CDPS WET Test Report Form when reporting data from the test (e.g. ~~A~~Excess receiving water toxicity observed. Division approved use of performance control for statistical comparisons per 5/30/96 phone call with Sarah Daphnia.®)

Reference Toxicant
Tests

Refer to the EPA guidance manuals for reference toxicity testing. It is required that the permittee keep track of QA/QC and reference toxicity test results.

Alternative Test Methods

Artificial ammonia
toxicity

The toxicity of ammonia is due mostly to its un-ionized fraction which is primarily a function of the temperature and the pH of the water being tested. As the pH and temperature increase so does

the toxicity of a given concentration of ammonia. In static WET tests, the pH in the test concentrations often increases (drifts) due to the loss of carbon dioxide (CO₂) from the test concentrations as they are incubated over the 24-hr period that they are used in the test. This upward drift results in pH values in the test concentrations that often exceed those pHs that could reasonably be expected to be found in the effluent under ambient conditions or in the mixing zone. Un-ionized ammonia toxicity caused by pH drift is considered to be an artifact of test conditions and is not a true measure of the ammonia toxicity likely to occur as the discharge enters the receiving waters.

In order to reduce the occurrence of artifactual un-ionized ammonia toxicity, it may be necessary to control the pH in toxicity tests. Control of pH should be done in a manner that has the least influence on the test water chemistry and on the toxicity of other pH sensitive materials such as some heavy metals, sulfide and cyanide. The only method that the Division may allow to control test pH is the use of the CO₂ amended atmosphere modification of chronic and acute test procedures. The adjustment of pH by other means such as the addition of acids or bases is not allowed.

The intended purpose of the CO₂ modification is to maintain the chronic test pH at or near the expected target instream pH at the end of the mixing zone (IWC dilution) and to maintain the acute test pH at or near the target end-of-the-pipe pH of the effluent taken at the time of sample collection. It is not the intended purpose, however, to maintain the test pH at unrealistically low levels which could reduce or eliminate true toxicity.

Before modified testing is undertaken, a permittee must demonstrate, to the Division's satisfaction, that artificial un-ionized ammonia toxicity due to pH drift has, or will, occur. This demonstration will include, but not be limited to, site-specific studies to determine the expected instream pHs for chronic and acute toxicity and the corresponding target pHs to be used in the CO₂ amended testing. Before any modified testing is conducted it must be approved by the Division through a permit modification.

A method for pH control testing is not given in the EPA guidance, but a generalized method has been presented in the literature (*A Simple Method Of pH Control For Static And Static-renewal Aquatic Toxicity Tests* by David R. Mount and Donald I. Mount, *Environmental Toxicology and Chemistry*, Vol. 11, pp. 609-614, 1992). Test containers can be exposed in a glass test box or in a incubator containing a CO₂ enriched atmosphere.

During the acute or chronic test, the pH shall be allowed to rise to the target test pH(s) specified above and should be maintained as close as possible to the target pH. After the first 24-hrs, the pH of test solutions must not be reduced by more than 0.3 units from the

target test pH during the remainder of the test. The test pH shall be monitored and controlled in the IWC dilution for chronic tests and in the 100% effluent in the acute tests. Use of the modified procedure will require additional pH monitoring in all other concentrations in the dilution series.

Data Analysis

Acute tests

Calculate the LC50 according to methods in Section 11 of *Methods for Measuring the Acute Toxicity of Effluents and Receiving Water to Freshwater and Marine Organisms - Fourth Edition* (EPA/600/4-90/027F August 1993).

Chronic tests

Follow the statistical methods presented in *Short-Term Methods For Estimating The Chronic Toxicity Of Effluents And Receiving Water To Freshwater Organisms - Third Edition* (EPA-600-4-91-002 July 1994).

This edition of the EPA chronic testing guidance now requires the use of the original number of fish starting the test to calculate the fathead minnow larval growth. Because this change could alter the outcome of some chronic tests, the Division will compare the results using the early method of calculation based on fish surviving the test. Therefore, results of fathead minnow tests should be reported based on per surviving and on per original basis. Lethality should also be reported because CDPS WET policy specifies this endpoint.

Summary of Acute Test Conditions

	<i>Ceriodaphnia</i>	Fathead Minnow
Test type:	static-renewal	static-renewal
Duration:	48 hours ("1 hour)	96 hours ("1 hour)
Effect measured:	death ¹	death ¹
No. of test concentrations ²	6, as a percentage of effluent	6 as a percentage of effluent
No. of replicate containers per concentration:	4 (minimum); randomize position of containers	4 (minimum); randomize position of containers
No. of organisms per container:	5 (exactly); randomly placed among containers	10 (exactly); randomly placed among containers
Test container size:	30 ml (minimum)	250 ml (minimum)
Volume of test solution per container:	15 ml minimum; uniform volume in all replicates	200 ml minimum; uniform volume in all replicates
Test solution replacement frequency:	daily	daily
Age of test animals:	less than 24 hours; from monoculture; minimum of 8 young per brood.	1-14 days; 24-h range in age
Feeding:	none (during test); feed neonates until they are	0.1 ml containing approximately 700 to 1000 placed in test
Temperature:	20 " 1EC (average daily)	20 " 1EC (average daily)
Light:	ambient laboratory levels (50 to 100 ft-c); 16 h light and 8 h dark	ambient laboratory levels (50 to 100 ft-c); 16 h light and 8 h dark
Aeration:	gently aerate effluent sample if dissolved oxygen falls below 4 mg/liter before	gently aerate effluent sample if dissolved oxygen falls below 4 mg/liter before making diluti

¹ Death is defined as: (1) no movement of gills or appendages; and (2) no reaction to gentle prodding.

² Select a minimum of 6 concentrations of effluent (volume/volume) from the following series: 100%, 75%, 50%, 25%, 12.5%, 6%, 0% (control). All test series must include a 100% effluent concentration and a 0% effluent concentration (control). For those situations where the Instream Waste Concentration (IWC) is less than 6% effluent, the dilution series may be modified in order to obtain toxicity information about the effluent toxicity at concentrations equal to or less than the IWC. This can be accomplished by substituting the IWC dilution for one of the intermediate dilutions, or by substituting an extra dilution, which is less than the IWC, for one of the intermediate dilutions. Likewise, when the IWC is greater than 50 %, a test concentration of 75% and/or the IWC should be included in the test series.

Summary of Chronic Test Conditions

	<i>Ceriodaphnia</i>	Fathead Minnow
Test type:	Static-renewal	Static-renewal
Duration: whichever occurs first	60% of control animals produce 3 broods	7 days (" 2 h) or at the end of 8 days,
Effect measured:	Decrease in reproduction; death ¹	Decrease in growth (weight); death ¹
No. of (minimum) ² test concentrations	6, as a percentage of effluent	6, as a percentage of effluent
No. of containers per concentration:	10 (minimum) ; randomized position of containers	4 (minimum) ; randomized position of (replicates) containers
No.of organisms per container:	1 (exactly); randomly placed among containers containers	10 (exactly) ; randomly placed among
No. of animals per concentration:	10 minimum, equally distributed among replicates replicates	40 minimum, equally distributed among
Test container size:	30 ml (minimum) equivalent to 500ml container	500 ml (minimum) or 300 ml if surface area is
Volume of test solution per container:	15 ml, uniform volume in all replicates	250 ml, uniform volume in all replicates
Test solution replacement frequency:	Daily	Daily
Age of test organisms:	Less than 24 hours; neonates must have been released within 8 hours of each other, from 3rd or subsequent broods of 8 or more; monoculture	< 24 hrs (if shipped then < 48 hrs); 24 h range in age
Feeding:	0.1 ml/day of trout chow, cerophyll and yeast (TCY) diet and 0.1 ml/day of <i>Selenastrum</i> suspension containing 3×10^7 to 4×10^7 cells/ml	0.1 ml containing approximately 700 to 1000 brine shrimp (<i>Artemia</i>) nauplii, three times a day at four-hour intervals or longer or 0.15 ml twice a day at six-hour intervals or longer
Temperature:	25 " 1EC (average daily)	25 " 1EC (average daily)
Light and photoperiod:	Ambient laboratory levels (50 to 100 ft-c); 16 h light and 8 h dark light	Ambient laboratory levels (50 to 100 ft-c);16 h and 8 h dark

(Chronic test conditions continued)

Aeration:	Aerate effluent sample if dissolved oxygen falls below 4 mg/liter before dilutions are prepared, none during test.	Aerate effluent sample if dissolved oxygen falls below 4 mg/liter before dilutions are prepared. If dissolved oxygen approaches 4.5 mg/l during testing, then gently aerate containers.
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- 1 Death is defined as: (1) no movement of gills or appendages; and (2) no reaction to gentle prodding.
- 2 Select a minimum of 6 concentrations of effluent (volume/volume) from the following series: 100%, 75%, 50%, 25%, 12.5%, 6%, 0% (control). All test series must include a 100% effluent concentration and a 0% effluent concentration (control). For those situations where the Instream Waste Concentration (IWC) is less than 6% effluent, the test series must be modified in order to obtain toxicity information about the effluent toxicity at concentrations equal to or less than the IWC. This can be accomplished by substituting the IWC dilution for one of the intermediate dilutions, or by substituting an extra dilution, which is less than the IWC, for one of the intermediate dilutions. Likewise, when the IWC is greater than 50 %, a test concentration of 75% and/or the IWC should be included in the test series.

COLORADO - CDPS WET TEST REPORT FORM - ACUTE

PERMITTEE: _____ CDPS NO. CO- _____ OUTFALL: _____

TYPE TEST: ROUTINE: _____ ACCELERATED: _____ TEST SPECIES: _____

TEST RESULTS: LC50 : _____ % STATISTICAL METHOD : _____

TEST TIME & DATE: BEGIN _____ AM/PM / ___ / ___ END _____ AM/PM / ___ / ___

DILUTIONS (% EFFLUENT)

MEASUREMENTS _____ CONTROL(0%) _____ % _____ % _____ % _____ %
_____%

NO. @ START OF TEST: _____

NO. LIVE AFTER 24 HRS: _____

AFTER 48 HRS: _____

AFTER 72 HRS: _____

AFTER 96 HRS: _____

RECEIVING WATER USED FOR DILUTION? YES / NO TOT. RESID. CHLORINE, MG/L: 100% _____

HARDNESS, MG/L: RECEIVING WATER _____ EFFLUENT _____ RECON WATER _____

ALKALINITY, MG/L: RECEIVING WATER _____ EFFLUENT _____ RECON WATER _____

PH: INITIAL - CONTROL _____ 100% _____ FINAL - CONTROL _____ 100% _____

T. AMMONIA AS N, MG/L: INITIAL - 100% _____ FINAL - 100% _____

WERE ALL TEST CONDITIONS IN CONFORMANCE WITH DIVISION GUIDELINES? YES / NO

IF NO, LIST DEVIATIONS FROM TEST SPECIFICATIONS: _____

LABORATORY: _____ ANALYST: _____

_____ COMMENTS: _____

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COLORADO - CDPS WET TEST REPORT FORM - **CHRONIC**

PERMITTEE: _____ CDPS NO. CO-00 _____ OUTFALL: _____

TEST TYPE: ROUTINE _____ ACCELERATED : _____ TEST SPECIES: _____ IWC: _____

TEST RESULTS: LETHALITY GROWTH REPRODUCTION

CONC. WITH STAT. DIFFERENCE	%	%	%
	PASS / FAIL	PASS / FAIL	PASS / FAIL
IC25	% <td style="text-align: center;">% <td style="text-align: center;">% </td></td>	% <td style="text-align: center;">% </td>	%
	PASS / FAIL	PASS / FAIL	PASS / FAIL

STAT. METHOD USED : STATISTICAL DIFF: _____ IC25: _____

DATE: START _____ AM/PM / / END _____ AM/PM / /

DILUTIONS (% EFFLUENT)

MEASUREMENTS _____ CONTROL (0%) _____ % _____ % _____ % _____ % _____

% SURVIVAL FOR DAY: 1	_____	_____	_____	_____	_____	_____
2	_____	_____	_____	_____	_____	_____
3	_____	_____	_____	_____	_____	_____
4	_____	_____	_____	_____	_____	_____
5	_____	_____	_____	_____	_____	_____
6	_____	_____	_____	_____	_____	_____
7	_____	_____	_____	_____	_____	_____

MEAN 3 BROOD TOTAL: _____

7-D MEAN DRY WT (starting): _____

7-D MEAN DRY WT:(survive): _____

PH MAX / MIN _____

HARDNESS (x) MG/L RECEIVING WATER: _____ EFFLUENT: _____ RECON WATER: _____

ALKALINITY (x) MG/L RECEIVING WATER: _____ EFFLUENT: _____ RECON WATER: _____

T. AMMONIA as N (x) MG/L INITIAL EFFLUENT: _____ FINAL EFFLUENT: _____

T. RESIDUAL CHLORINE, MG/L 100%: _____ RECEIVING WATER USED FOR DILUTION? YES / NO

WERE ALL TEST CONDITIONS IN CONFORMANCE WITH DIVISION GUIDELINES? YES / NO

IF NO, LIST DEVIATIONS FROM TEST SPECIFICATIONS: _____

LABORATORY: _____ ANALYST: _____

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