
DIVISION OF WATER QUALITY

NORTH CAROLINA DEPARTMENT OF
ENVIRONMENT AND NATURAL RESOURCES

# NORTH CAROLINA *CERIODAPHNIA* CHRONIC WHOLE EFFLUENT TOXICITY PROCEDURE

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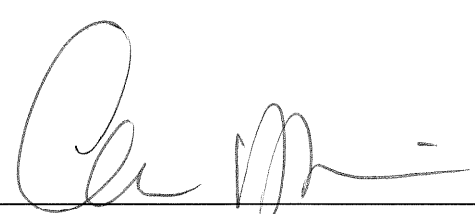

4/21/11

**Coleen H. Sullins, Director, Division of Water Quality** **Date**

# North Carolina *Ceriodaphnia* Chronic Whole Effluent Toxicity Procedure (*Ceriodaphnia* Mini-Chronic Toxicity Test)

This procedure has been established as a modification of Method 1002.0 described in the U.S. Environmental Protection Agency document entitled "Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms," Fourth Edition (EPA-821-R-02-013). This procedure has been reduced in complexity in order to provide a relatively inexpensive means of assessing suitable water quality with respect to chronically toxic substances.

The test as outlined within the main section of this document may be used as a routine monitoring tool and yields data that shows the presence or absence of toxicity of a solution. It does not determine the no-effect level. At times, estimating the actual no-effect level may be necessary in order to evaluate the degree of toxicity reduction needed. This may be performed using an expanded series of dilutions. Additions to the methodology outlined here which will estimate the no-effect level are outlined in the Appendix 2.

This test procedure has been approved by the Director of the North Carolina Division of Water Quality under the Certification Criteria/Procedures document described in the Biological Laboratory Certification Rules (15A.NCAC.2H.1100) as a standard procedure for evaluation of the effects of toxic substances to sensitive aquatic species. It shall be considered acceptable proof that the effluent is not causing impacts to aquatic life in the receiving streams due to toxic substances. It does not directly address mutagens, carcinogens, teratogens or disease causing agents and may be superseded by other water quality regulations. Depending on the use designation and specialized concerns of a particular water body (or effluent discharge), additional monitoring and/or restrictions (either chemical or biological) may be required. These monitoring requirements may include, but are not limited to, additional toxicity testing using alternate test organisms, unmodified EPA protocols and increased sampling or test solution renewal frequencies.

The test organism used for this pass/fail test is *Ceriodaphnia dubia*, a small cladoceran common in lakes and larger rivers and frequently used as an aquatic toxicity test organism. The organism has a rapid life cycle at 25°C, potentially producing numerous offspring during a seven day period. This particular test method is not used to determine a no-effect level of the effluent discharge; it is intended to evaluate whether the discharge toxicity is acceptable or unacceptable in relation to a pre-established No-Observed-Effect Level (NOEL).

The measures of effect used within this test are number of offspring produced and mortality within the test period. This document will outline approved variations from the EPA procedure. Only those modifications outlined here or in the North Carolina Biological Laboratory Certification/Criteria Procedures Document or approved by written exception made by NC DWQ may be made to the EPA guidelines. This document is organized into six sections which include:

1) Effluent sampling and handling,
2) Test procedure,
3) Interpretation of results,
4) An outline of daily activities to be performed prior to and during the test period (Appendix 1),
5) Methodology to estimate the actual effluent NOEL (Appendix 2), and
6) Quality Assurance Checklist (Appendix 3).

## EFFLUENT SAMPLING AND HANDLING

All effluent samples collected for this procedure must be 24-hour composites unless grab samples or other alternate sampling regimes are specifically allowed by the facility's permit or monitoring requirement. Sampling should be performed below the last waste treatment process, including disinfection. There may be no removal of chlorine or any other effluent constituent by either chemical or physical methods prior to testing with the exceptions of allowable filtration of the effluent through 60 µm nylon screen or plankton netting and reduction of excess dissolved oxygen to the saturation level, as per EPA methods.

Sample collection materials may be tempered glass, polyethylene, perfluorocarbon plastics including Teflon®, 304 or 316 stainless steel, polypropylene, polyvinylchloride, Tygon®, or silicone. All non-perfluorocarbon plastics should be discarded after use. It is the responsibility of the sample collector to assure that contamination is not influencing test results. There may be no chemical residue present which will affect effluent toxicity. Care should be taken that sufficient volume is collected in order to perform the test.

Effluent samples must be immediately preserved on ice such that they achieve and maintain a temperature between 0.0°C and 6.0°C, inclusive, from collection, in the case of grab samples, or initiation of collection through the use of an iced or refrigerated sampler, in the case of composite samples. The single allowable exception to this protocol is the situation in which a grab sample arrives at the performing laboratory within three hours of collection. In that circumstance, the sample container must be completely covered in ice in its shipping container immediately after collection and arrive at the laboratory in that same condition. All other samples must be received by the certified biological laboratory at a temperature between 0.0°C and 6.0°C, inclusive, or the resulting data will be considered unacceptable for submittal for compliance purposes. The sample container must be completely filled, with no air pocket, to minimize loss of volatiles. Aliquots only should be drawn from the original sample for warming and subsequent use in tests.

Each effluent sample collected for this procedure must follow certain timing/scheduling constraints. By definition of this method, each composite sampling must be performed over two calendar days (Day One through Day Two, and Day Three through Day Four, as defined in Appendix 1). For purposes of defining the month in which the test is indicative of compliance, the start date of the first sample for any given test will be considered the month (and quarter) in which the test was performed. The sampling schedule is intended to be performed on Monday through Tuesday and Wednesday through Thursday. Shifting the sampling days is acceptable, assuming that the relative chronology and sequence of sampling and testing activities remains constant and the certified biological laboratory is capable of meeting such a schedule. Effluent samples for chronic tests are to be first used within 36 hours of collection and not more than 72 hours after first use for test renewal. Sample holding time begins at the time of collection of a grab sample or the time of collection of the last subsample of a composite sample, and ends when the organisms are introduced or transferred to the test solution. First use of the sample is defined as the time the last organism is introduced to the test solution when initiating/renewing a test. Samples must be stored at 0.0-6.0°C, with minimum head space. For example, a composite sample initiated on Monday at 10:00 AM and terminated at 10:00 AM on Tuesday must be used for the first time by 10:00 PM on Wednesday. Likewise, the second sample, initiated at 10:00 AM on Wednesday and terminated at 10:00 AM on Thursday, must be used for the test renewal by 10:00 PM on Friday. The second sample must be used for the final test renewal not more than 72 hours after first use. As such, careful coordination should take place between sampling personnel and the certified biological laboratory so that sampling schedules can be accommodated within protocol constraints of the testing method.

Preparation of split samples must be performed carefully to insure that each laboratory receives and analyzes similar samples. This similarity should take into account possible variables including, but not limited to, sample mixing, sample containers, lack of air space in sample containers, sample temperature, pH, conductance, and total residual chlorine. Additionally, if concurrent analyses are sought on split samples, performing laboratories should coordinate analytical times and dates. Analyses of split samples performed at significantly different times or on different dates will be considered as independent analyses.

## TEST PROCEDURE

The test shall be performed as two treatments exposing 12 test organisms to each treatment. The first treatment shall be considered the control population and shall be exposed at 0% effluent and 100% dilution water. Dilution water must be the culture water used to maintain the test population or be suitable for that purpose. This treatment will be used to evaluate the significance of effect in treatment two. The pH of the control solution at test initiation and subsequent test solution renewals must fall in the range of 6.5-8.5 standard units. Total hardness must measure between 30 and 50 mg/l $CaCO_3$. Treatment two will be (unless specified otherwise) a concentration of effluent diluted by the dilution water to the following percent:

$$\%\ \boldsymbol{Effluent}\ (\boldsymbol{IWC})^{\blacklozenge} = \frac{\text{Permitted Discharge Volume} * 100}{\text{Permitted Discharge Volume} + 7Q10^{\blacksquare}}$$

♦ *Treatment Two.*

■ *Where 7Q10 is defined as the lowest average 7-day flow in the receiving stream which has a probability of reoccurrence every ten years. All terms must have equivalent units.*

Twelve test organisms will be exposed to each treatment in individual test chambers. The test will run until at least 80% of the surviving female control organisms produce three broods of young, not to exceed a seven day + 2 hours exposure, using the chronology specified in Appendix 1. Termination prior to seven days should also be contingent upon whether the control reproduction mean has reached the minimum acceptable value of 15.0 young per surviving female.

The objective of this test is to determine whether treatment two, which exposes the test population to an effluent concentration equal to the wastewater's concentration in the receiving stream during low stream flow conditions, has significant detrimental impact upon reproduction and survival as compared to the control population (treatment one). If there is no significant detrimental impact compared to the control population then the effluent is not considered chronically toxic to instream inhabitants and is considered to have passed the test. A failure will be considered as either: 1) a significant decrease in survival of the treatment organisms as compared to the control organisms or 2) a twenty percent or greater decrease in treatment organism reproduction which is also determined to be significantly different from control organism reproduction.

After effluent collection on Days One through Two, the test treatments will be established and the test initiated on Day Three (Appendix 1). An aliquot of the first composite sample is brought to room temperature and utilized to mix test solutions which are then distributed to the test vessels. The specific conductance, pH, and total residual chlorine of the undiluted effluent sample must be measured and recorded. (Effluent samples are to be refrigerated at a temperature between 0.0° and 6.0° C except for those aliquots drawn for mixing test solutions.) The pH, dissolved oxygen and temperature of the control and test treatment must be checked and recorded. At all times test solution temperature must be 25.0°C (±1.0°C) and dissolved oxygen levels must be greater than or equal to 5.0 mg/l. The test organisms are

placed singly in test vessels each containing 15 ml of solution. The organisms must be less than 24 hours old, within 8 hours of the same age, from third or subsequent broods, and from broods in which the adult produced at least 8 neonates. The test organisms must be produced by "individual" cultures as defined by "Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, Fourth Edition" (EPA-821-R-02-013). Neonates are transferred using an eye dropper, such that the organism is never removed from solution. There should be as little water transferred with the organism as is reasonably practical. The *Ceriodaphnia* should be fed at this time and daily thereafter. Each daily feeding will consist of addition of 0.05 ml of yeast-Cerophyll®-trout chow (YCT) food and 0.05 ml of a solution of the algae *Selenastrum capricornutum* (with a cell concentration of $1.71 \times 10^7$ cells/ml) per 15 ml of test solution. Preparation of food supplies are described by "Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Fourth Edition" (EPA-821-R-02-013) (alternative algal media preparation methods are described in "North Carolina Biological Laboratory Certification/Procedures Document"), though feeding rates have been modified for this protocol. Test chambers should be incubated for temperature control with the photoperiod maintained at 16 hours of light and 8 hours of darkness.

On Days Three through Four, a second effluent composite is collected to be used for renewal of the test solutions on Days Five and Eight. On Day Five the original test organisms are transferred to new test vessels containing new solutions of treatment one (control) and treatment two (effluent concentration). The effluent solution is mixed from the second effluent sample collected on Day Four. The specific conductance, pH, and total residual chlorine of the undiluted effluent sample must be measured and recorded. This renewal must take place within 36 hours of the second effluent sample collection time.

Mortality should be recorded at this time. Should mortality in treatment two significantly exceed that of treatment one, as determined by Fisher's Exact Test, the test may be terminated and the effluent sample declared a failure due to significant mortality. Reproduction counts should be performed in all vessels used during the initial test period (although there are usually no offspring during this phase in the life cycle). Temperature, dissolved oxygen, and pH observations must also be made and recorded for both the old and new test solutions. The new test solutions should receive food at this time.

Days Six and Seven require only that the *Ceriodaphnia* be fed. Day Eight requires renewal of the test solutions using the second effluent sample. This renewal must take place within 72 hours of the first use of the effluent sample on Day Five. Mortality, reproduction, temperature, dissolved oxygen and pH observations must be made and recorded. Reproduction of the initial test organisms must be observed both as total number of young produced as well as brood number of the young produced (i.e. first, second or third brood). As stated previously, the test may be terminated if significant mortality has occurred in the effluent treatment (treatment two). On Day Nine the control organisms should be observed for production of the third brood. If 80% or more of the surviving control organisms have produced a third brood, the test may be terminated. This will also hold true for observations made on Day Eight. On Day Ten, the test is terminated after making final mortality, reproduction and temperature, dissolved oxygen, and pH measurements. Fourth brood neonates will be excluded from the reproduction totals and subsequent statistical analyses. The test exposure duration will be no greater than seven days + 2 hours regardless of control organism reproductive success. All entries to test bench sheets should be initialed by the person making the entry in a manner that will signify which entry was made by which analyst.

## INTERPRETATION OF RESULTS

The statistical comparisons for evaluating the test results will be performed as outlined in "Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, Fourth Edition" (EPA-821-R-02-013) with the exception that reproduction data are to be evaluated at a 99% confidence level. The statistical methods used should be those in the EPA document Appendix H titled "Single Concentration Toxicity Test - Comparison of Control with 100% Effluent or Receiving Water." A statistically significant twenty percent or greater reduction in neonate reproduction by the treatment organisms as compared to the control organisms or statistically significant mortality among the treatment organisms will be considered a failure of this effluent to meet toxic standards within the receiving stream. Mortality greater than 20% in the control population will be considered as abnormal and the test must be repeated. Reproduction in the control population must be greater than or equal to 15.0 offspring per surviving female as an average of total reproduction. No more than 20% of the control organisms may be males. For the purpose of determining reproductive suppression of the treatment organisms as compared to the controls, mean reproduction is calculated by summing the total number of young produced through three broods per treatment until either time of death or end of the experiment and dividing by the initial number of females exposed per treatment. Percent reduction will be calculated by subtracting the mean number of neonates produced by the treatment organisms from the mean number of neonates produced by the control organisms, dividing that number by the mean number of neonates produced by the control organisms, and multiplying by 100% as per the following equation:

$$\boldsymbol{Percent\ Reduction} = \left(\frac{\bar{Y}_1 - \bar{Y}_2}{\bar{Y}_1}\right) * 100\%$$

Where $\bar{Y}_1$ is the control organism reproduction mean and $\bar{Y}_2$ is the treatment organism reproduction mean. Note that fourth brood neonates will be excluded from the reproduction totals.

The control organism reproduction coefficient of variation (CV) must be less than 40.0% for the test to be considered acceptable. The CV is calculated by dividing the standard deviation of the control organism reproduction by the mean of the control organism reproduction multiplied by 100 % as per the following equation:

$$\boldsymbol{Coefficeint\ of\ Variation\ (CV)} = \left(\frac{s_1}{\bar{Y}_1}\right) * 100\%$$

Where $s_1$ is the standard deviation and $\bar{Y}_1$ is the mean. Note that the mean and standard deviation are calculated using the number of female organisms initially exposed to the control solution, including any which may have died during the course of the test.

If these tests are being performed as an NPDES requirement or by Administrative Letter, then data must be entered on the Effluent Discharge Monitoring Form (MR-1) for the month in which it was performed using the appropriate parameter code. Additionally, DWQ Form AT-1 (original) is to be received at the following address no later than the last day of the month following the month in which the analysis occurs:

Environmental Sciences Section
Aquatic Toxicology Unit
North Carolina Division of Water Quality
1621 Mail Service Center
Raleigh, North Carolina 27699-1621

# APPENDIX 1. MINI CHRONIC TOXICITY TEST

**Day One**

This test procedure, including sampling, has been designed to minimize weekend work if begun on a Monday. A 24-hour compositing device will be started. Sampling devices should be refrigerated or cooled by ice. The final sample volume should be a minimum of 500 milliliters.

**Day Two**

The composite sample will be collected, sealed, and packaged on ice to maintain a temperature between 0.0° and 6.0°C, inclusive, and shipped to the laboratory where the toxicity test will be performed. (Alternatively, a grab sample may be collected on this day if the NPDES permit specifies such a sample.)

**Day Three**

The test treatments will be set up and test organisms introduced within 36 hours of sample collection time on Day Two. Dissolved oxygen, temperature and pH will be measured and recorded. Dissolved oxygen must be greater than or equal to 5.0 mg/l and the temperature must be maintained at 25.0°C (±1.0°C). The specific conductance, pH, and total residual chlorine level of the undiluted sample must be measured and recorded. Minimize head space of the effluent sample and refrigerate (0.0°-6.0°C). Feed *Ceriodaphnia.* Start second 24-hour effluent composite sample.

**Day Four**

Collect and ship the second effluent sample. Feed *Ceriodaphnia*.

**Day Five**

*Ceriodaphnia* must be transferred to new solutions of the second effluent sample prior to the sample reaching 36 hours in age. The specific conductance, pH, and total residual chlorine level of the undiluted effluent sample must be measured and recorded. Record the time at which the test organisms are transferred. Mortality and reproduction counts should be performed at this time (although there are usually no offspring during this early phase of the life cycle). Perform temperature, dissolved oxygen, and pH monitoring. Feed *Ceriodaphnia*. Minimize head space of the second effluent sample and refrigerate (0.0°-6.0°C).

**Day Six**

Feed *Ceriodaphnia*.

**Day Seven**

Feed *Ceriodaphnia.*

**Day Eight**

Renew all test solutions using second composite sample. Test solutions must be renewed within 72 hours after first use of the sample on Day Five. Record the time at which the test organisms are transferred. Count mortality and reproduction. Perform temperature, dissolved oxygen, and pH monitoring. Feed *Ceriodaphnia*.

**Day Nine**

Feed *Ceriodaphnia* (Optional: Observe reproduction and terminate test if greater than 80% of surviving control organisms have produced a third brood).

**Day Ten**

Perform final mortality and reproduction counts as well as temperature, dissolved oxygen, and pH monitoring.

## APPENDIX 2. METHODOLOGY TO ESTIMATE THE ACTUAL NOEL USING THE NORTH CAROLINA PHASE II CHRONIC WET PROCEDURE

In order to estimate a no-effect level, the *Ceriodaphnia* are exposed to a series of effluent concentrations. This contrasts with the above procedure which uses only one concentration and a control. The reproduction within each effluent concentration is subject to a statistical analysis as defined in "North Carolina Phase II Chronic Whole Effluent Toxicity Test Procedure, Version 3.0" July 1991, Revised December 2010 to determine the chronic value. If the chronic value is greater than the previously defined pass/fail concentration, then the test is reported as a "Pass."

Procedures for performing this analysis are described in "North Carolina Phase II Chronic Whole Effluent Toxicity Test Procedure, Version 3.0" July 1991, Revised December 2010. All effluent sampling, test conditions, and test procedures are identical to those outlined in the main section of this document except for the test concentrations, number of organisms per treatment, and statistical evaluations of data. Ten organisms will be used for each treatment. The analysis of these data will be performed using methods described in the document cited above. Note that the statistical evaluation of reproduction effects will be based on a 95% confidence level.

## APPENDIX 3. QUALITY ASSURANCE CHECKLIST

The following table summarizes appropriate test conditions for the NC mini-chronic toxicity test. Values recorded outside of these ranges will result in an analysis being judged as "invalid" upon review by Aquatic Toxicology Unit personnel. The information should be used as a checklist for individual tests and does not cover the full range of quality control practices necessary for successful completion of this analysis.

| TEST CONDITION | TEST ACCEPTABILITY CRITERION |
|---|---|
| Effluent % | By Permit, SOC, or JOC |
| Control Mortality | ≤20% |
| Average Reproduction for Control | ≥15.0 per surviving female |
| % Control Organisms Producing a Third Brood | ≥80 % surviving controls |
| Maximum % Male Control Organisms | ≤20% |
| Control Reproduction CV | <40.0 % |
| Initial Control Solution pH | 6.5-8.5 pH units |
| Minimum D.O. of Control and Treatment 2 | ≥5.0 mg/l |
| Hardness Dilution Water | Between 30-50 mg/l $CaCO_3$ |
| Sample Temperature at Receipt | Between 0.0-6.0°C |
| Temperature during Test | 25.0 ± 1.0°C |
| First Use of Sample for Test Initiation and/or Solution Renewal | <36 Hours from sample collection |
| Subsequent Use of Sample for Test Solution Renewal | <72 hours after first use of sample |

## REFERENCES

North Carolina Division of Water Quality. 2010. North Carolina Biological Laboratory Certification/Criteria Procedures Document, Version 3.0. Revised December 2010.

North Carolina Division of Water Quality. 2010. North Carolina Phase II Chronic Whole Effluent Toxicity Test Procedure, Version 3.0. July 1991, Revised December 2010.

United States Environmental Protection Agency. 2002. Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Fourth Edition. EPA 821-R-02-013, 350 pp.

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